

Award Number: DAMD17-01-1-0365

TITLE: Molecular Determinants of tGolgin-1 Function

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REPORT DATE: July 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20031114 079

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 2003	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jul 02 - 30 Jun 03)	
4. TITLE AND SUBTITLE Molecular Determinants of tGolgin-1 Function			5. FUNDING NUMBERS DAMD17-01-1-0365	
6. AUTHOR(S) Atsuko Yoshino, Ph.D. Michael Marks, Ph.D. Mark Lemmon, Ph.D.			8. PERFORMING ORGANIZATION REPORT NUMBER	
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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) <p>tGolgin-1 is a large, predominantly coiled coil peripheral membrane protein that associates with the <i>trans</i> Golgi network (TGN) by virtue of a C-terminal GRIP domain. We showed that overexpression of isolated GRIP domains from tGolgin-1 or related proteins results in disruption of the TGN by inhibiting recycling from endosomes, suggesting that tGolgin-1 and other GRIP domain proteins function in this pathway. This is important because signalling molecules required for initiating transformation and for metastasis rely on sorting and processing in the TGN. In collaboration with Dr. Chris Burd, we showed that GRIP domain localization to the TGN in yeast is regulated by a small GTPase cascade, in which the Arf-like protein Arl3 recruits the Arf-like protein Arl1, and activated Arl1 in turn binds directly to and recruits GRIP domains. Orthologues of these proteins function similarly in mammalian cells. Using RNAi to ablate expression of tGolgin-1 from mammalian cells, we have shown that tGolgin-1 also participates in the association of the Golgi complex to the dynein/dynactin complex. This has important implications for a role of tGolgin-1 in cell motility and polarization required for tumor development, metastasis, and immunity. We are now probing for the mechanism of this function of tGolgin-1.</p>				
14. SUBJECT TERMS Intracellular protein transport, trans golgi network, protein processing, golgin			15. NUMBER OF PAGES 76	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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INTRODUCTION

This report summarizes the training and research accomplishments over the second year of funding from award # DAMB17-01-1-0365. The award supports research directed toward understanding the function of tGolgin-1 (also known as golgin-245, trans golgi p230, and 256 kD golgin), a peripheral membrane protein associated with the *trans* Golgi network (TGN) of mammalian cells (Erlich et al., 1996; Fritzler et al., 1995; Gleeson et al., 1996). tGolgin-1 is a member of a family of large peripheral membrane proteins that are predicted to consist largely of coiled-coil structure with a small predicted globular region of homology at the C-terminus referred to as the GRIP domain. The GRIP domain was shown by several groups to mediate localization of these proteins to the cytosolic face of the Golgi complex and/or TGN (Barr, 1999; Brown et al., 2001; Kjer-Nielsen et al., 1999; Luke et al., 2003; Munro and Nichols, 1999). A function for tGolgin-1 or any of the other GRIP domain proteins has not yet been described. In addition to the GRIP domain, tGolgin-1 is predicted to contain a small globular domain at the N-terminus.

BODY

We had shown that GRIP domain proteins contribute to the integrity of the TGN and to protein sorting and processing events that occur within the TGN, likely through regulating recycling of TGN resident proteins from endosomal compartments. This was done by overexpressing isolated, epitope-tagged GRIP domains from either tGolgin-1 or golgin-97, which competed for binding of endogenous GRIP domain proteins from the Golgi complex. This displacement was associated with defects in resident integral membrane protein localization to the TGN and in TGN structure and function. Among those proteins that require GRIP-dependent TGN modifications and sorting functions are growth factor receptors associated with breast cancer induction, such as epidermal growth factor receptor and Her2, matrix metalloproteinases required for metastasis of breast tumors, and tumor antigens analogous to the melanoma-associated antigen Pmel17/gp100. Thus, GRIP domain overexpression may influence the growth and metastasis of tumors; moreover, dissecting how GRIP domain protein structure and function are related may reveal novel approaches for treatment of breast tumors. The goals of the experiments as proposed in the *Statement of Work* were to elucidate such structure/function relationships by dissecting functional domains of tGolgin-1 and by characterizing their interacting partners.

Since the inception of the funding from this grant, we have made significant progress in shoring up our preliminary data, advancing our abilities to tackle the originally proposed aims in the *Statement of Work*, and progressing in some new and very exciting directions. In the context of these advances and published work from other groups, the aims in the *Statement of Work* were modified at the end of the last funding period. Below we detail our progress.

1. Sequence and expression of mouse tGolgin-1. In the first year, we completed an initial characterization of the mouse tGolgin-1 cDNA and the expression of the corresponding mRNA in different tissues. These findings were published in this funding period (Cowan, D *et al.*, 2002. DNA and Cell Biol., **21**: 505-517; **see appendix for reprint**). Dr. Yoshino is a co-author of this work.
2. Role of GRIP domain proteins in maintenance of the TGN. This study was largely completed in the first year, and a manuscript describing this work is in press in *The Journal of Cell Science* (Yoshino et al, 2003. *J. Cell Sci.*, in press). Dr. Yoshino is the first author on this work. The main findings of the study are that overexpression of GRIP domains results in (1) displacement of endogenous TGN resident proteins, but not residents of the Golgi stack or other subcellular organelles, to peripheral membranes, including multivesicular late endosomes; (2) vacuolization of the Golgi and the TGN; (3) disruption of protein transport of the vesicular stomatitis virus G protein from the Golgi to the plasma membrane; and (4) disruption of furin-dependent cleavage of a substrate protein. We speculate that this implicates a role for GRIP domain proteins or GRIP domain ligands in regulating the recycling of TGN resident proteins from endosomal compartments back to the TGN. **See appendix for a copy of the accepted manuscript.**

3. Functional analysis of tGolgin-1. As discussed in the last Progress Report, these studies were initiated during the first funding period (2001-2002) as the result of emerging technology using inhibitory RNAs (RNAi; (Elbashir et al., 2001; Elbashir et al., 2002)), and have become a major focus of the project. Dr. Yoshino found that transfection of cells with small inhibitory RNA duplexes (siRNA) corresponding to part of the coding region near the 5' end of the tGolgin-1 sequence resulted in depletion of endogenous tGolgin-1 in transfected cells. Under optimal conditions, Dr. Yoshino has successfully reduced expression of endogenous tGolgin-1 to below detection limits in 80-90% of transfected cells as judged by immunofluorescence microscopy (to 10% of normal levels in the population of transfected cells as judged by western blotting). Initial analyses of these cells by immunofluorescence microscopy showed that markers of the TGN were dislocated to peripheral punctate structures as described in last year's Progress Report. Further analyses have shown that the defects in these cells extend beyond the TGN. Indeed, the entire Golgi complex becomes fragmented, and reforms as fully functional "mini Golgi" elements at specific exit sites of the endoplasmic reticulum; these sites can be marked by the accumulation of a GFP-tagged form of Sec13, a component of the COPII coat involved in ferrying cargo molecules from the endoplasmic reticulum to pre-Golgi intermediates. Interestingly, this is the same phenotype observed in cells in which microtubules are disrupted by treatment with drugs such as nocodazole (Cole et al., 1996), or in cells in which the minus-end directed motor protein complex, dynein/dynactin, is disrupted by overexpression of the p50 dynamitin subunit (Burkhardt et al., 1997). As in cells with disrupted microtubules or dynein/dynactin complexes, secretory transport proceeds with at best minimal kinetic delay. These results thus imply that tGolgin-1 is required for anchoring the Golgi complex to dynein/dynactin motors. Dr. Yoshino has shown that this requirement is likely due to an indirect mechanism rather than through direct binding of dynein/dynactin subunits to tGolgin-1. This was done by following the movement of pre-Golgi intermediates and of TGN elements, including tGolgin-1, toward the microtubule organizing center in cells recovering from brefeldin A; pre-Golgi elements and tGolgin-1-containing membranes both moved toward the center of the cell, but independently. We hypothesize that the inability to link the Golgi to dynein/dynactin in cells lacking tGolgin-1 expression is the result of a recycling defect, such that a critical dynein recruitment protein fails to be retrieved from post-Golgi membranes back to the Golgi complex. If verified, this would support a role for tGolgin-1 in such a recycling step and lend further credence to our results with overexpressed GRIP domains. Preliminary results following the retrograde movement of bacterial toxins supports this notion, but the results need to be confirmed and extended. This work, for which a manuscript on which Dr. Yoshino is first author is currently in preparation, is important in showing a requirement for the TGN in positioning the Golgi complex. Such positioning may be critical for a number of biologically important functions relevant to breast cancer formation, metastasis and treatment such as maintenance of cell polarity, the ability of cells to undergo directed migration, and the ability of cytotoxic T lymphocytes to kill target cells. While Dr. Yoshino continues to probe the mechanism by which tGolgin-1 functions, others in the lab are now assessing these potential functional outcomes of ablated tGolgin-1 expression.

4. Molecular determinants of tGolgin-1 function. This was the main thrust of the work proposed in the *Statement of Work*. As discussed in last year's progress report, we have altered these aims based on published work and work from our collaborators. Significant progress has been made, and we plan to continue to focus on this aim as well as on Aim 3.

Task 1 was to characterize membrane complexes containing endogenous tGolgin-1 or its derivative N-terminal or GRIP domains. *Task 2 and 3* were to identify the requirements for tGolgin-1 binding to the TGN membrane via the GRIP domain, and to endosomal membranes via the N-terminal domain, respectively. As discussed in last year's Progress Report, we changed the focus toward understanding the regulation of GRIP domain interactions with the small GTPases, Arl1 and Arl3, and toward better defining the function of the N-terminal domain.

In collaboration with Dr. Chris Burd (Univ. of Pennsylvania), we used yeast genetics and in vitro assays with purified proteins to probe the relationship between Arl1, Arl3, and GRIP domain localization to the yeast TGN. This work, published earlier this year in *Current Biology* (Setty et al., 2003. *Curr. Biol.* **13**: 401-404; **see appendix for reprint**), showed that activation of Arl3p was required to recruit Arl1p to TGN membranes, and that GTP-activated Arl1p then recruited GRIP domains to TGN membranes. The

latter interaction was shown to be direct, as recombinant GRIP domains could bind to recombinant Arl1p in the presence of a non-hydrolyzable form of GTP. This work was supported by another concurrent publication (Panic et al., 2003), and a recent publication by the Hong group (Lu and Hong, 2003) shows that the Arl1p-GRIP domain interaction is conserved in mammalian cells. Given the advantages of the yeast system and the ability to generate recombinant yeast (Imh1p) GRIP domains in bacteria but not recombinant GRIP domains from tGolgin-1 or golgin-97, we plan to pursue this part of *Task 1* through collaboration with the Burd laboratory.

To analyze the N-terminal domain of tGolgin-1, Dr. Yoshino has generated mammalian expression constructs and bacterial fusion proteins corresponding to the N-terminal globular domain without or with an adjacent coiled-coil region that is highly conserved between the mouse and human orthologues. When expressed in mammalian cells, both fragments localize to discrete structures, suggesting that they are capable of binding to some stable macromolecular structure within cells. We are now using the recombinant proteins and a new antibody that we generated to characterize additional molecules that bind to the N-terminal domain using in vitro binding assays and immunoprecipitation, respectively. We hope to identify specific interacting proteins through mass spectrophotometric sequencing techniques.

The original aim also included studies analyzing the potential binding of GRIP domains to phosphatidylinositides and other lipids. In several attempts to assay for such an interaction, we have been unable to observe binding of recombinant GRIP domains to any cellular lipid using a sensitive dot blot assay that is commonly employed in the Lemmon laboratory. Furthermore, Biacore analyses of phosphatidylinositide binding of recombinant yeast Imh1p GRIP domains have yielded no positive results. We thus believe that our initial hypothesis that GRIP domains bind to lipids was incorrect.

Additional Training

Dr. Yoshino continues to develop as an independent researcher, extending a trend she began in the previous funding period. She developed Aims 3 and 4 above nearly completely independently of Drs. Marks and Lemmon, and as a result has made a very significant contribution to the field. Dr. Yoshino has gained experience supervising technical staff, successfully directing a Research Specialist over the last year, and is now helping to supervise a graduate student who is following up on some of her findings. She has learned a number of new approaches in the last year, including cell fractionation, biochemical purification of bacterial fusion proteins, real-time fluorescence microscopy and associated quantitative techniques, and analyses of cytoskeletal elements. Furthermore, she has delved into a new literature – motor-dependent movement on microtubules – and has become proficient at reading, interpreting, and conveying these important studies. She has continued to improve her presentation skills in group meetings with five labs, in small group meetings of the Marks lab alone, and in personal interactions with members of the Marks, Lemmon, and Burd laboratories. Dr. Yoshino has presented her work at meetings of the Japanese Biochemical Society in 2001, 2002, and 2003, and at a Gordon Conference in 2001.

KEY RESEARCH ACCOMPLISHMENTS

- Completion of analysis of GRIP domain overexpression (manuscript in preparation)
 - mislocalization of TGN resident integral membrane proteins
 - vacuolization of the TGN
 - disruption of TGN function (sorting to plasma membrane and furin-dependent proprotein processing)
 - disruption of endosome to TGN recycling
- Functional analysis of tGolgin-1 using siRNA and live cell imaging
 - dissociation of Golgi complex from microtubule organizing center
 - reformation of functional “mini-Golgis” at endoplasmic reticulum exit sites
 - indirect requirement of tGolgin-1 for minus-end-directed movement of Golgi fragments on microtubules
 - failure to detect interactions between tGolgin-1 and dynein/ dynactin components
- Identification of Arl-1 as ligand for GRIP domains in yeast
- Initial characterization of tGolgin-1 N-terminal domain interactions with cellular factors

REPORTABLE OUTCOMES

1. Published manuscript: D. Cowan, D. Gay, B.M. Bieler, H. Zhao, **A. Yoshino**, J.G. Davis, M.M. Tomayko, R. Murali, M.I. Greene and M.S. Marks (2002). Characterization of mouse tGolgin-1 (golgin-245/ trans golgi p230/ 256kD golgin) and its upregulation during oligodendrocyte development. DNA and Cell Biol. **21**: 505-517.
2. Published manuscript: S.R.G. Setty, M.E. Shin, **A. Yoshino**, M.S. Marks and C.G. Burd (2003). Golgi recruitment of GRIP domain proteins by Arf-like GTPase 1 (Arl1p) is regulated by Arf-like GTPase 3 (Arl3p). Curr. Biol. **13**: 401-404.
3. Manuscript in press: **A. Yoshino**, B.M. Bieler, D.C. Harper, D.A. Cowan, S. Sutterwala, D.M. Gay, N.B. Cole, J.M. McCaffery and M.S. Marks (2003). A role for GRIP domain proteins and/or their ligands in structure and function of the *trans* Golgi network. J. Cell Sci., in press.
4. Manuscript in preparation: **A. Yoshino**, C. Poynton, C.G. Burd, E.L. Holzbaur, J.M. McCaffery and M.S. Marks. A TGN function mediated by tGolgin-1 is required for Golgi tethering to cytoplasmic dynein. In preparation.
5. Abstract for Meeting of the Japanese Biochemical Society, 2002: **A. Yoshino**, B.M. Bieler, D.C. Harper, D. Cowan, D. Gay, J.M. McCaffery and M.S. Marks. Regulation of TGN protein localization by GRIP domain proteins.
6. Abstract for Meeting of the Japanese Biochemical Society, 2003: **A. Yoshino**, J.M. McCaffery and M.S. Marks. Characterization of the function of TGN-localized peripheral membrane protein, tGolgin-1.

CONCLUSIONS

Our research thus far has assigned a function to tGolgin-1 and potentially other GRIP domain proteins, a group of peripheral Golgi-associated proteins that have been described for a number of years but for which no function has been known. These proteins appear to be involved in a critical step of protein transport from endosomes to the TGN, a subcellular organelle required for protein sorting and modification within both the secretory and endocytic pathways. Disruption of this recycling pathway affects TGN function – such as delivery of critical growth factor receptors and proprotein cleavage of matrix metalloproteinases and other critical metastatic factors. Furthermore, tGolgin-1 is required for

positioning the Golgi at the microtubule organizing center. Disruption of its function is thus anticipated to affect cell polarity, migration, and polarized secretion. The gene encoding tGolgin-1 has been shown to be deleted in at least one lung carcinoma (Ishikawa et al., 1997), suggesting that manipulation of its function may be important for tumorigenesis. Continued work on the function and physical interactions of tGolgin-1 and other GRIP domain proteins may therefore provide us with insights into manipulation of tumors. More directly, elucidating the function of these mysterious peripheral membrane proteins will help us to understand the biology of intracellular protein transport.

As discussed above, future work will focus on elucidating the cellular function of tGolgin-1 and other GRIP domain proteins using RNAi approaches, and on identifying interacting factors with the N-terminal domain of tGolgin-1 as a means of elucidating its mechanism of action.

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APPENDICES

1. Manuscript reprint: Cowan , et al., 2002 (13 pages: pages 10-22).
2. Manuscript reprint: Setty, et al., 2003 (4 pages: pages 23-26).
3. Accepted manuscript in press: Yoshino, et al., 2003 (49 pages: pages 27-75).
4. Abstract from Meeting of the Japanese Biochemical Society, 2003 (1 page: page 76)

Characterization of Mouse tGolgin-1 (Golgin-245/*trans*-Golgi p230/256 kD Golgin) and Its Upregulation during Oligodendrocyte Development

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ABSTRACT

As part of an effort to identify gene products that are differentially regulated during oligodendrocyte development, we isolated a mouse cDNA that encodes tGolgin-1, a homolog of the human protein known as golgin-245, *trans*-golgi p230, or 256 kD golgin. Human tGolgin-1 is the target of autoantibodies in patients with Sjögren's syndrome, and is thought to be involved in vesicular transport processes at the *trans*-Golgi network. Sequencing of cDNAs and EST clones comprising the full-length tGolgin-1 transcript predict marked homology with the amino- and carboxy-terminal regions of the human protein, but more limited homology within the central predicted coiled-coil region. Epitope tagged, truncated forms of mouse tGolgin-1, like those of its human homolog, were localized at steady state to the Golgi/*trans*-Golgi network in transfected cells. The tGolgin-1 message was expressed in all tissues examined, but was highly upregulated in oligodendrocyte precursors at a stage just prior to myelination. This expression pattern suggests that tGolgin-1 may play a role in specialized transport processes associated with maturation and/or differentiation of oligodendrocyte precursors.

INTRODUCTION

OLIGODENDROCYTES ARE RESPONSIBLE for the highly complex process of myelination within the central nervous system. Oligodendrocytes arise from undifferentiated neuroepithelial precursors within the ventricular zone of the spinal cord (Orentas and Miller, 1998). These precursors undergo a complex developmental program during which they differentiate first into highly proliferative, pluripotent migratory cells, and then into nonmotile, immature oligodendrocytes that make direct physical contacts with target neurons (Pfeiffer *et al.*, 1993; McMorris and McKinnon, 1996). Mature oligodendrocytes synthesize myelin components, such as myelin basic protein, myelin-associated glycoprotein, myelin/oligodendrocyte protein, and proteolipid protein, which are incorporated into myelin-rich regions of the plasma membrane that enwrap axons of adjacent neurons (Campagnoni, 1988). These developmental changes are marked by the expression of distinct cell surface markers, particularly glycolipids or glycoproteins, and are accompanied by dramatic changes in cellular morphology

and physiology. These changes include the extension of cell processes, changes in expression of adhesion molecules and growth factor receptors, and ultimately, the generation and compaction of myelin sheets at great distances from the cell body (Pfeiffer *et al.*, 1993).

The developmental changes in the physiologic and morphologic characteristics of oligodendrocyte precursors likely reflect alterations in the cytoskeleton and in the biosynthetic machinery of the cell. Indeed, unusual microtubule arrays (Lunn *et al.*, 1997) and peripheral Golgi-like structures (de Vries *et al.*, 1993) have been found in the extended processes of oligodendrocyte precursors. Furthermore, oligodendrocytes at later stages of development display a number of features characteristic of polarized cells (Pfeiffer *et al.*, 1993), such as differential sorting of viral glycoproteins (de Vries *et al.*, 1998). The generation of these unusual morphologic features and protein sorting pathways must be orchestrated within the cytoplasm and the central vacuolar system by structural and regulatory proteins, the expression of which is also likely to be developmentally regulated. For example, Rab3a and Rab3c, proteins respectively as-

sociated with synaptic and endocrine secretory vesicles, are up-regulated during later oligodendrocyte development (Madison *et al.*, 1996), and a specialized Rab protein, Rab22b, is expressed selectively in oligodendrocyte lineage cells (Rodriguez-Gabin *et al.*, 2001). It would be expected that Rab effector proteins and other components of the trafficking machinery would also be differentially regulated in these cells.

We previously described a novel cell surface determinant, termed OIP-1 (Oligodendrocyte Precursor protein-1), that is specifically expressed on developing oligodendrocyte precursors at a stage just prior to the onset of synthesis of myelin components (Gay *et al.*, 1997). In an effort to characterize the OIP-1 antigen, we used an anti-OIP-1 monoclonal antibody (mab) to screen a cDNA expression library from a postnatal day 8 mouse brain. Using this approach, we identified a set of cDNA clones that encode partial, alternatively spliced forms of a single gene product. Sequence analysis of cDNAs spanning the entire coding region identified the gene product as the murine homolog of a human protein previously identified as golgin-245 (Fritzler *et al.*, 1995), *trans*-golgi p230 (Kooy *et al.*, 1992), or 256 kD golgin (H. P. Seelig, Genbank accession #X82834) and referred to here as tGolgin-1. tGolgin-1 is a peripheral membrane protein of unknown function found on the cytosolic face of the *trans*-Golgi network (TGN) and associated vesicles (Gleeson *et al.*, 1996). It is part of a family of peripheral membrane proteins containing a large, central coiled-coil region and a C-terminal "GRIP" domain that is responsible for localization to the Golgi and/or TGN (Barr, 1999; Kjer-Nielsen *et al.*, 1999a, 1999b; Munro and Nichols, 1999; Brown *et al.*, 2001). tGolgin-1 mRNA was ubiquitously expressed, but was dramatically upregulated in oligodendrocyte precursors at the stage of development marked by expression of the OIP-1 determinant. We postulate that upregulation of tGolgin-1 during this stage of development facilitates either the establishment of peripheral Golgi-like structures in oligodendrocyte processes or the synthesis, targeting, or recycling of myelin constituents in preparation for myelin formation.

MATERIALS AND METHODS

Animals and tissue culture conditions

Virus-free BALB/c postnatal day 1 mice (Harlan, Indianapolis, IN) provided cerebral tissue for all primary oligodendrocyte and astrocyte cultures. Oligodendrocyte precursor culture conditions were as described in Gay *et al.* (1997). Cerebral cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) for 2–4 weeks to generate enriched astrocyte populations. The BALB/C7 fibroblast and HeLa cell lines (ATCC) were maintained in DMEM/10% FBS.

cDNA library production, screening, and sequencing

mRNA was extracted from brain tissue of postnatal day (P)8 mice using the Fastrack system (Invitrogen, Carlsbad, CA), converted into cDNA and directionally cloned using the ZAP Express cDNA II cloning kit (Stratagene, La Jolla, CA). Recombinant fusion proteins were screened for binding to anti-OIP-1 mab with the Picobule Immunoscreeing Kit (Stratagene). These original cDNAs were sequenced using automated se-

quencing facilities and T3, T7, and internal designed primers. Sequences were analyzed using Geneworks and BLAST software. Additional regions were characterized following identification of mouse EST clones with homology to regions of the human p230 cDNA further 5' than the original mouse cDNA clones. EST clones with accession numbers (Acc) AA144704, AA561361, and AA389589 were obtained from Genome Systems Inc. (St. Louis, MO). The region between EST clone AA144704 and cDNA clone C91 was amplified by reverse transcriptase (RT) coupled polymerase chain reaction (PCR) from neonatal mouse brain RNA as described (Marks *et al.*, 1995) and cloned using unique NheI and PshAI restriction sites. The region encompassing EST clone AA098411 up to the NdeI site of EST clone AA144704 was also generated by RT-PCR from neonatal mouse brain RNA. The region corresponding to the coding region for the N-terminus of human p230 was generated by RT-PCR from adult mouse brain and liver mRNA using a degenerate oligonucleotide encoding the N-terminal human p230 residues and a reverse primer downstream of the NdeI site. The sequence of the 5' UTR and the translation start site were inferred from overlapping genbank database entries (Acc BC003268, BF162586, BI151932, and BE304004). See Figure 1 for schematic diagram. Oligonucleotide sequences are available upon request. Sequences of amplified regions were confirmed from at least three cloned products of at least two separate RT-PCR reactions, and only sequences confirmed by two or more database entries are listed for the 5' UTR. Coiled-coil predictions were done using the MTIDK matrix within the COILS v2.2 program from the Swiss Institute for Experimental Cancer Research (http://www.isrec.isb-sib.ch/software/COILS_form.html).

Northern analysis

RNA was isolated from the indicated tissues of female Balb/c mice using the FastTrack 2.0 kit (Invitrogen) for poly(A)+ RNA or the RNA STAT-60 kit (Tel-Test, Inc., Friendswood, TX) for total RNA. RNA samples (25 µg total or 2.5 µg polyA+) were fractionated on 0.8–0.9% agarose gels in formaldehyde/MOPS essentially as described (Sambrook *et al.*, 1989). Gels were soaked in 0.05 M sodium hydroxide for 20 min, then 20× SSC for 1 h prior to transfer onto Immobilon Ny+ filters (Millipore, Bedford, MA) overnight in 20× SSC. Blots were washed in DEPC-treated H₂O, and then baked at 80°C for 2 h. Digoxigenin-labeled probes were generated by PCR using the PCR DIG Probe Synthesis Kit from Boehringer Mannheim-Roche (Palo Alto, CA), and hybridized to filters and developed using the DIG Luminescent Detection Kit according to the manufacturer's instructions. Bands were visualized using a Molecular Dynamics (Sunnyvale, CA) Storm 760 Phosphor Imager and ImageQuant v. 1.1 software. The probe for tGolgin-1 encompassed nt. 110–1172, and the probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) encompassed the entire open reading frame (nt. 47 to 1045).

HA-tagged constructs

HA11 epitope tag sequences were constructed by PCR for ligation onto the N- or C-terminal coding regions of the clone C91 cDNA sequence. For an N-terminal tag, a PCR product including a 5' Kozak consensus sequence, the coding region for an HA11 tag, and a region contiguous with the 5' sequence for

C91 was subcloned into the SalI and SphI sites of C91 to generate HA-tGolgin-C1247. For a C-terminal tag, a PCR product contiguous with the coding region for the C-terminus of C91 and containing a 3' HA tag followed by a stop codon was cloned into the NruI and XbaI sites of C91 to generate tGolgin-C1247-HA. Resultant constructs were subcloned into the pCDM8.1 expression vector (Bonifacio *et al.*, 1990). Additional truncation products were generated by PCR from the original clones using similar designed primers, and all amplified products were verified by automated DNA sequencing.

Immunofluorescence microscopy

HeLa cells plated on glass coverslips in six-well dishes were transiently transfected using the calcium phosphate method (Sambrook *et al.*, 1989), fixed with 2% formaldehyde 40 h post-transfection, and processed for indirect intracellular immunofluorescence microscopy as previously described (Marks *et al.*, 1995). Antibodies included a rabbit antiserum to TGN46 (Prescott *et al.*, 1997) (a gift of V. Ponnambalam, Univ. Dundee), a rabbit antiserum to the β -COP subunit of COPI (a gift of Dr. J. Lippincott-Schwartz, National Inst. of Health, Bethesda, MD), anti-HA11 monoclonal antibody (BabCo, Richmond, CA) and secondary fluorochrome-coupled antibodies (Jackson ImmunoResearch, West Grove, PA). Slides were analyzed on a Zeiss Axioplan fluorescence microscope (Zeiss, Thornwood, NY), and photographic images were digitized using a Nikon LS-1500 film scanner. In one experiment, images were captured with a Leica TCS laser confocal scanning microscope equipped with a 100 \times , 1.4 N.A. oil immersion lens and the Leica TCS software package.

Flow cytometry and cell sorting

Precursors from day 4 (populations 1 and 2) or day 9 (population 3) mixed mouse oligodendrocyte cultures were incubated with combinations of anti-OIP-1 (Gay *et al.*, 1997), anti-O4, R-Mab (Ranscht *et al.*, 1982) and O1 (anti-GalC) mabs and prepared for cytofluorography as described (Gay *et al.*, 1997). Cell sorting was accomplished using a FACStar Plus (Becton Dickinson, Franklin Lakes, NJ) and CellQuest software.

Semiquantitative RT-PCR

mRNA was extracted from 10⁵ cells using Micro-FastTrack (Invitrogen), converted to cDNA with Superscript II reverse transcriptase (Life Technologies, Rockville, MD) and PCR-amplified using the High Fidelity PCR system (Roche Applied Science, Indianapolis, IN). Semiquantitative PCR was carried out on 5 \times 10³ cell equivalents with an α ³²P dCTP tracer using previously specified conditions (Tomayko and Cancro, 1998). Samples (2 μ l) were removed at designated cycles, electrophoresed onto a 0.8% agarose gel, vacuum dried and subjected to autoradiography. Nonquantitative PCR was carried out for 40 cycles using mRNA from approximately 10⁵ cells. The cDNA 1 PCR product was TA cloned and sequenced to show 100% identity to cDNA clone C91 (unpublished data). The actin primers were as specified (Tomayko and Cancro, 1998). The PLP primers were 5'gactacaagaccacatctg3' (nt. 309 to 328 and 5'gaagaggccaatcagt ggca3' (nt. 923 to 943). The cDNA 1 primers for tGolgin-1 were 5'ggctgggagagctgaggcaga3' (nt. 3260 to 3280) and 5'gttcaagctctgtctccag3' (nt. 3640 to 3660).

The cDNA 2 primers were 5'tgggtcagagacttcagact3' (nt. 4730 to 4750) and 5'tgtcagctctgggaatctg3' (nt. 5216 to 5235).

RESULTS

Isolation of a tGolgin-1 cDNA by screening a postnatal mouse brain cDNA library with anti-OIP-1

OIP-1 was identified as a developmentally regulated cell surface determinant specific to premyelinating oligodendrocyte precursors (Gay *et al.*, 1997). Because these cells are most highly represented in the mouse brain during early postnatal development, a postnatal day 8 mouse brain cDNA expression library was screened with anti-OIP-1 mab. Three independent cDNAs (clones C95, C49, and C91) were retrieved ranging in size from 2.2 kb to over 4 kb. Sequencing showed them to be identical except for a 63 bp deletion at the 3' end of the largest cDNA clone (C91). Figure 1a shows the relative sizes and sequence overlap of these cDNAs. The sequence of C91 contained a single open reading frame of 3741 nucleotides extending to the extreme 5' end. The 3' terminus was verified by the presence of multiple stop codons and a poly A tail.

Comparison of the predicted translation product of the C91 open reading frame to available databases identified three entries with considerable homology: golgin 245 (accession number U31906; Fritzler *et al.*, 1995), 256 kD golgin (acc. no. X82834), and *trans*-golgi p230 (acc. no. U41740) (Erllich *et al.*, 1996), corresponding to products of the *GOLGA4* gene. These three molecules bear identical nucleotide sequences except for deletions within the 3' and/or 5' regions, and we, therefore, believe that they represent the product of a single gene with a single alternative splice site near the 3' end (see Discussion). Because of their immunolocalization to the TGN (Gleeson *et al.*, 1996), we refer to them collectively as tGolgin-1. The region of homology extended through the entire predicted translation product of C91, but corresponded to only the C-terminal 1247 amino acids of the 2228–2230 residue full-length tGolgin-1 protein. This suggested that C91 was not a full-length cDNA for mouse tGolgin-1.

A BLAST search of the mouse EST database revealed numerous additional clones with predicted translated amino acid sequence similarity to regions within the N-terminal 1000 residues of human tGolgin-1. Two of these clones, Acc AA098411 and AA144704, were retrieved, and together spanned sequences encoding 878 additional contiguous residues N-terminal to those encoded by C91 (Fig. 1a). cDNA sequences between EST clone AA144704 and C91 were amplified by RT-PCR from postnatal mouse brain RNA, and EST clone AA098411 was reconstructed using RT-PCR from the same source using the available sequence deposited in Genbank. The coding region for the predicted N-terminal region was amplified by RT-PCR of prenatal mouse brain RNA using a degenerate primer corresponding to the coding region for the first six amino acids of human tGolgin-1 and a second reverse primer from within the coding region of EST clone AA144704. The remaining 5' UTR and coding region for the first six amino acids are inferred from overlapping entries in the Genbank database (see Materials and Methods for details). RT-PCR of mRNA from several sources indicated that all separately identified regions were colinear in a single RNA species (see below). We refer to this composite clone as mouse tGolgin-1.

Q L Q S Q V E K L E A D L G C S L S E K L S L Q E E L A E L 1175
 3601 CTCAGCTGCA GTCACAAAGT GAAAGCTGG AAGCTGATT GGGTGTCTCT CTGAGTGAAA AGCTTTCCCT TCAAGAGAG CTGGCTGAGC 3690
 K L L A D K S Q L R V S E L T G Q V Q A A E K E L Q S C K S 1205
 3691 TGAAGCTGCT GGCAGACAG AGTCAGCTGA GGTCTCCGA GCTGACGGG CAGGTGACAG CTGCGGAGAA AGCTGTAACT 3780
 L E E L S K K S L E D K S L N L K S L L E E L A S Q L D S R 1235
 3781 CTCTGCTGTA GCTCAGCAAG AAGAGCTGG AGGACAAGAG CTGTAACCTT AAAAGCTTGC TTGAGGAGCT AGCATCTCAG CTAGACATCT 3870
 C E R T K A L L E A K T N E L V C T S R D K A D A I L A R L 1265
 3871 GCTGTGAGAG AACCAAGCTT CTGTAGAAAG CCAAGACGAA CGAAGTATGT TGCACACGCG GTGACAAAGC CGACGCTATT CTGCTGAGGC 3960
 S Q C Q R H T A T V G E A L L R R M G Q V S E L E A Q L T Q 1295
 3961 TGTCCCAATG CCAAGCCAC ACAGCCACAG TTGGGGAGGC GCTGCTCAGG AGAATGGGCG AGGTTTCTGA GTTAGAGCA CAACCTTACAC 4050
 L T E E Q R T L K S S F Q Q V T N Q L E E K E K Q I K T M K 1325
 4051 AGCTGACAGA GGAGCAGCGC ACATAAAGA GCTCTTTTCA GCAGTTTACC AATCAGCTGG AAGAGAAAGA GAAGCAGATT AAGACCATGA 4140
 A D I E G L L T E K E A L Q Q E G G Q Q R Q A A S E K S C 1355
 4141 AGCTGACAT TGAAGTCTT CTACAGAAA AAGAAGCTT ACAGCAAGAA GGAGCCAGC AGCCGACAGC GGCCTCTGAG AAGGATCTCT 4230
 I T Q L K K E L A E N I N A V T L L R R E L S E K K S E I A 1385
 4231 GCATCAGACA GCTGAAGAAA GAGTACGGG AGACATCAA TGCCCTCACC CTACTGAGAG AAGAGCTGTC GGAGAAAGAG TCTGAGATTG 4320
 S L S K Q L S D L G A Q L L Q E S S I S P S D K A E A I S A L 1415
 4321 CCACTCTTGA AGTATCTCG ATGCTACGCT GAGAGCAGC ATCAGCCCGA GTGCAAGGC CGAAGCCCTA TCTGCCCTGA 4410
 K Q H E E Q E L Q L Q A Q L Q E L S L V D A L S K E K M S 1445
 4411 GCAAGCAGCA CGAGGACAAA GAGCTGACGC TGCAAGCTCA GCTCTCAGAG CTGTCTCTGA AAGTCGATGC TCTGAGTAAA GAGAAATGT 4500
 A L E Q V D H W S N K F S E N K K K A Q S R L A Q H Q S T I 1475
 4501 CTGCGCTTGA GCAAGTGGAT CATGTGCTCA ACAAGTTCTC AGAGTGGAGG AAAAAGACAC AGTCAGACT TGCCGACGAC CAAGACTTAA 4590
 K D L Q A Q L D V K A T D A R E K E E Q I C L L K E D L D R 1505
 4591 TTAAAGACTT GAGGACAGC CTAGACGTA AAGCCACGGA CGCTCGTGAG AAGGAGGAGC AGATATGTTT ACTGAAGAAA GACCTTGATC 4680
 Q N K K F E C L K G E M E V R K S K M E K K E C D L E T A L 1535
 4681 GGCAGATATA AAGATTGAA TGTTTAAAG GCGAGATGGA AOTCAGGAG AGCAAGATGC AGAAGAAAGGA GTGTGACTTA GAGACTGCGT 4770
 K T Q T A R V V E L E D C V T Q R K K E V E S L N E T L K N 1565
 4771 TAAAGCTCA GACAGCAAGG GTCGTGAAT TAGAAGACTG CTACTACGAG AGGAAAGAG AAGTTGAGTC CTTAATGAA ACACCTTAAG 4860
 Y N Q Q R D T E H S G L V Q R L Q H L E L G E E K D N K V 1595
 4861 ATTCAACCA GCAAGGGAGC ACTGAGCACA GTGGGCTGGT TCAGCACTTG CAGCACTTGG AAGAGTGGG AGAAGAGAAA GACACCAAGC 4950
 R E A E E T V L R L R E H V S S L E A E L G T V K K E L E H 1625
 4951 TTAGGAGGCG TGAAGAGACC GTCTGAGAG TACGAGACA CGTGTCTCTG CTGGAAGCTG AACTTGGAAC TGTGAAGAG GAACCTAGAC 5040
 V N S S V K S R D G E L K A L E D K L E L E S A A K V E L K 1655
 5041 ATGTAATTC AAGTGTGAAA AGCAGAGATG GGGAGCTGAA AGCTTTAGAA GACAAACTTG AGTTAGAAA TGTGCAAAA GTGAGCTGAA 5130
 R K A E Q K I A A I R K Q L L S Q M E E K T Q R I A K D T E 1685
 5131 AGAGGAAGGC CGAGCAGAG ATCGCTGCCA TCAGGAAGCA GCTCTGTCTC CAGATGGAAG AGAAGACCCA GCGGTATGCG AAGGACAGC 5220
 N R L S E L S A Q L K E R E K Q V H S L E D K L K N L E S 1715
 5221 AGAAGACTCT GAGTGAAGCT AGTCACAAAT TAAAGAAAG AGAAGAACAA GTTCACAGCC TAGAAGACAA ACTTAAAGAC CTGAGAGATT 5310
 P H P E V P A V S R S M Q S V A A S P E Q E A P D S C T 1745
 5311 CTCCACACCC AGAAGTGCCA GCGGTGCCA GATCTATGCA AAGTGTGCCA GCGTCTCCCG AGCAAGAGGC TCCAGATTCC CAGGACTGCA 5400
 H K A C K E R L C M L Q R R L S E K E K L L R R L E Q G E 1775
 5401 CACCAAGGC CTGTAAAGAA AGACTGTCCA TGCTGCAAG ACCTTTAAGT GAAAGAGAGA AGCTGCTGCG CAGGCTGGAG CAGGGCAGAG 5490
 K A R P S Q P E A Q R A L S G K L D C T R A R Q L E H V 1805
 5491 GCGAGGCGAG GCGATCGCAG CCGTAGGCTC AGCAGCAGGC GCTCTCTGGA AAGTTAGACT GCACTAGAGC CAGGACAGCT GAAGCTACAG 5580
 L I G C L P E E L E E K N K C S L I V S Q P M N E T G N N 1835
 5581 TTTGTATAGG ATGCTTCCA GAAGAATCG AAGAAAGAT GAAATGTTC TTAATTTGCT CTCAGCCAT GGGAGAGAAA ACTGTTACA 5670
 T G V K Q N W A S V V D S V Q K T L Q E K E L T C Q A L E 1865
 5671 ACACAGGGGT GAGCAGAAAT TGCGCAAGTG TGTGTGACAG TGTTCAGAAA ACCCTCCAGG AAAAGAGCT CACTTGGCC GCCTAGAGC 5760
 R V K E L E S D L V R E R G A H R L E V E K L T L K Y E K S 1895
 5761 AAAGGCTGAA AGAAGCTGAG TCAGACTTGA TAAGAGAGAG GGGGCCCAT AGACTTGAAG TGGAAAAGTT GACCTTAAA TATGAAAT 5850
 Q S S Q Q E M D G E N K C V E V L E D R P E E S S Q S H E I 1925
 5851 CGCAATCTTC CCAAGCAAG AAGATGAGG AAAATAAATG TGTAGAAAGT TTGAAGACA GGCCTGAGGA AAACCTCCAA TCCCATGAGA 5940
 Q S N V G T V D G L R S D L E S K L T G A E R D K Q K L S K 1955
 5941 TCCAGTCAAG COTGGGAGC GTGGACGGCC TGCGCAGCGA CCTGGAATCC AAGTTGACAG GAGCGGAGCG GGACAAGCAG AGCTGAGCA 6030
 E V A R L Q K E L R A L R R E S Q Q E L D I L K R E C E Q E 1985
 6031 AGGAGGTGCG GAGGCTCAG AAGGAGCTCG GAGCTCTGCG GAGGAGCAC TGACATCTCT TGACATCTCT GAAGAGGAGG TCGGAGCAG 6120
 A E E K L K Q E Q E D L E L K H T S T L K Q L M R E F N T Q 2015
 6121 AGCCGAGGA AAACTCAAA CAGGACCAAG AGGATCTTGA OTTGAAGCAC TGAAGCAGCT TGAAGCAGCT GATCGGGAG GATTACACAC 6210
 L A Q K E Q E L E R T V Q E T I D K A Q E V E A E L L E S H 2045
 6211 AACTGGCACA GAAGGAGCAG GAGCTGGAAA GAACCTGCTA GGAGACCAT GATAAGGCC AAGAGGTGGA AGCCGAACT CTGGAAGAGC 6300
 Q E E T Q Q L H R K I A E K E D D L R R T A R R Y E I L D 2075
 6301 ACCAAGAGA GACACAGCAG TTGCATAGA AGATCGCGGA GAAAGAGGAT GATCTGAGAA GGACAGCCAG GAGATACGAG GAGATACCTG 6390
 A R E E E M T G K V T D L Q T Q L E E L Q K K Y Q Q R L E Q 2105
 6391 ATGCCCCGTA AGAAGAAATG ACTGGCAAG TGACCGAGCT CGACAGCCAG CTGAGGAGC TCCAGAGAA ATACAGCAG AGGCTGGAGC 6480
 E S T K D S V T I L E L Q T Q L A Q K T T L I S D S K L K 2135
 6481 AGGAGGAGAG CACCAAGGAC AGTGTAAACA TTTTGGAGCT ACAGACACAA CTAGCGCAGA AGACCACTCT GATCAGCAG TCCAAGCTGA 6570
 E Q L R E Q V H N L E D R L K R Y E K N A C A A T V G T P 2165
 6571 AGGAGGAGAG GTTGAGAGAA CAGGTCCATA ATTAGAAGA CCOTTTGAAA AGATATGAAA AGAATGCATG TGCAGCAACT GTGGGAGAC 6660
 Y K G H L Y H T E V S L F G E P T E F E Y L R K V M F Y 2195
 6661 CTACAAAGG TGGCAATTTG TACCACACT AGGTCTCACT CTTCGAGAAA CCTACCGAGT TTGAGTATT GCGAAAAGTG ATGTTTGAAT 6750
 M M G R E T K T M A K V I T T V L K F P D D Q A Q K I L E R 2225
 6751 ATATGATGG TCGGAGACT AAGACCATGG CCAAGTTAT AACCACTGTC CTGAAATTC CTGATGATCA GGCTCAGAAA ATTTTGAAA 6840
 E D A R L M S W L R T S S * 2238
 6841 GAGAGATGC TCCTGTGAT TCATGGCTCC GAACCTCATC TTGAagaat ggagagagtg agctaggcag cgcgtgcctg ggaagctgtc 6930
 6931 caactgtgggc ttttagagaa tggcgctgtc attacagcca ttgggttagc ttgaaggcag atgtactctt ggcctcagat acattgtgtt 7020
 7021 ggaagcaggc gcttttggtt ggggatgtgt ggtgggtttt tctcttttta ttcagctttt tcttggaag tttctgtgtt ttttagagct 7110
 7111 gttctgactg gcccacactg gcttggtgtt tctctgtgtt tctctctctt ttttagaagc tgtccatctt ctttagattt 7200
 7201 ttttaggtgt tttctttttt ttcttaaga cttgtgcaat atatttttag gtgaaactta gtgaattttt ttctgataaa tttagatttt 7290
 7291 taattgactg tttaacagat tgattttttt atattttgtt aaagcctagt tctttatgtc atgataagtc ccaaatggaa cttactatgt 7380
 7381 tttactagtc ttttgactt atattatttt ttagaggaat aaactactat gtaantgtta aatagatcat aatcctattt tctgagcttc 7470
 7471 tgtgactgtt ggccatgtca tcttagagga ttagataaat aaagtatttt tctctaaaaa 7530

FIG. 1. (Continued) Isolation and sequence of mouse tGolgin-1 cDNA. (a) Schematic representation of regions of sequence overlap between *trans*-golgi p230, golgin-245, 256 kD golgin, isolated OIP-1 cDNA clones (C91, C49, and C95), EST clones (AA144704, AA561361, and AA389589), and additional regions identified in this work. The triangle represents the 63 bp insertion in p230 and clones C95 and C49. 1 and 2 represent the locations of PCR products used in Figure 7. Regions identified by RT-PCR only are indicated. Database represents the 5' UTR and translation start sequences that were deduced from the deposited sequences of Genbank entries BC003268, BF162586, BI151932, and BE304004; these sequences were not confirmed by our direct sequencing, but represent overlap of the corresponding database entries. (b) Nucleotide sequence of mouse tGolgin-1 cDNA and deduced amino acid sequence. Italics represent the inferred sequences from the database entries as indicated above.

The complete cDNA sequence and deduced amino acid sequence of mouse tGolgin-1 is listed in Figure 1b, and the deduced amino acid sequence is compared with that of human tGolgin-1 in Figure 2A. The mouse tGolgin-1 open reading frame encodes 2238 amino acids with a predicted molecular mass of 258 kDa, compared to 2230 amino acids and 261 kDa for the human homolog. The predicted amino acid sequences of the two homologs show 70% identity and 85% similarity. Interestingly, *trans*-golgi p230 and 256 kD golgin, like mouse tGolgin-1 cDNA clones C49 and C95, maintained the 65 bp 3' nucleotide stretch encoding "FTSPRSGLF" at the C-terminus, whereas golgin-245 and clone C91 had this region deleted, generating an alternative "SWLRTSS" C-terminal end. Both human and mouse proteins are predicted to consist primarily of coiled-coil domains, with short regions of potentially globular structure at the N- and C-termini (Fig. 2B).

To determine whether the sequences we identified corresponded to the only mouse homolog of human tGolgin-1, we searched the dbest database for homologous murine EST sequences using BLAST. We retrieved 145 nucleotide sequences that were greater than 93% identical to mouse tGolgin-1, most of which were mismatched at only one to three residues; given the distribution of the nucleotide differences, we suspect that these clones contain identical sequences to those in mouse tGolgin-1 and that differences reflect sequencing errors and/or potential haplotype variations. Included among the retrieved sequences were clones homologous to regions spanning the entire mouse tGolgin-1 cDNA, with the exception of the short adenosine-rich stretch between C91 and AA144704. Searches of the dbest database for translated sequences homologous to either the human tGolgin-1 proteins or the translated mouse tGolgin-1 sequence resulted in no additional entries other than those identified on the basis of nucleotide similarity. This suggests that no other tGolgin-1 homologues have yet been entered in the EST database. The fact that they were obtained from highly diverse cDNA library sources suggests that they represent an ubiquitous mRNA, as would be expected for a tGolgin-1 homolog (see below).

Significant but low homology was also observed between the mouse tGolgin-1 deduced amino acid sequence and the C-terminal half of myosin II (unpublished data). This homology, which ranged from 23–45% over four distinct regions, correlated specifically with predicted coiled-coil regions as assessed using the MTIDK matrix (Lupas *et al.*, 1991) (Fig. 2B). The GCG computer program "Moment" used to calculate helical dipole moments to predict α -helical regions failed to find long helices within the tGolgin-1 sequence (unpublished data). These data cannot distinguish between the possibilities that tGolgin-1 is supercoiled like the myosin heavy chain C-terminal domain or globular with many small coiled-coil-like domains. However, because myosin II and several other coiled-coil containing Golgi- and endosome-associated peripheral membrane proteins are believed to form dimeric supercoiled structures, we anticipate that tGolgin-1 has a similar structure.

tGolgin-1 localization to the TGN through the C-terminal half of the molecule

Because human tGolgin-1 has been shown to be peripherally associated with the TGN (Gleeson *et al.*, 1996), we assayed for the intracellular localization of the mouse tGolgin-1 C-terminal fragment encoded by C91. Expression vectors encoding

HA11 epitope-tagged forms of the translation product of C91 (HA-C1247, representing the C-terminal 1247 aa of tGolgin-1) were transiently transfected into HeLa cells, and localization was determined by indirect intracellular immunofluorescence microscopy (IFM) using anti-HA11 antibodies. As shown in Figure 3, HA-C1247 localized to a paranuclear, reticular structure reminiscent of the Golgi complex. Parallel staining with an antiserum to the endogenous TGN resident protein, TGN46 (Fig. 3A and B), or to the Golgi stack-associated β -COP subunit of coatomer (Duden *et al.*, 1991) (Fig. 3C and D) showed extensive overlap, suggesting that the C-terminal tGolgin-1 fragment primarily localizes to the TGN and/or the Golgi stacks. Similar results were obtained using C1247 tagged at the C-terminus with the HA11 epitope (unpublished data). Like human tGolgin-1 (Gleeson *et al.*, 1996), the TGN-associated HA-C1247 was partially dispersed upon treatment with brefeldin A (unpublished data). These data further support the notion that we identified the mouse homolog of human tGolgin-1, and that tGolgin-1 localizes to the TGN.

The C-terminal 80 amino acids of human tGolgin-1 encompass a GRIP domain, a modestly conserved region that is necessary and sufficient to mediate localization to the TGN (Barr, 1999; Kjer-Nielsen *et al.*, 1999a, 1999b; Munro and Nichols, 1999). To determine whether the comparable region of mouse tGolgin-1 was sufficient to mediate Golgi localization, we generated N-terminally HA-tagged truncation mutants containing the C-terminal 312, 186, or 81 amino acids of mouse tGolgin-1, and expressed them by transient transfection in HeLa cells. As shown in Figure 4a–f, immunofluorescence microscopy analyses indicated that all of these constructs colocalized well with TGN46. Mutagenesis of a critical conserved tyrosine residue at position 2187 completely disrupted localization, resulting in a diffuse cytoplasmic staining pattern (Figure 4g and h). These results indicate that mouse tGolgin-1 retains a functional GRIP domain, and that the C-terminal 81 amino acids are sufficient to specify localization to the Golgi/TGN.

Expression of mouse tGolgin-1 mRNA: abundant expression in oligodendrocyte precursors

The distribution of human tGolgin-1 mRNA in different tissues has not been evaluated. To assess the tissue distribution of mouse tGolgin-1, RNA was prepared from a panel of mouse tissues and hybridized with a probe derived from the coding region of the mouse tGolgin-1 cDNA (Fig. 5). tGolgin-1 mRNA migrates as a single major band of approximately 7.5 kb in length, as expected from the length of the predicted full-length cDNA and by analogy with the human tGolgin-1 mRNA. Overexposure of the blot reveals additional bands from brain tissue at approximately 9 and 12.5 kb, which may represent incompletely spliced heteronuclear RNAs or alternative splice products; the 9-kb band was only observed in brain tissue (unpublished data). The 7.5-kb mRNA is expressed in all tissues analyzed, as expected for a gene product involved in constitutive transport processes. Relative to the GAPDH controls, no clear enrichment was observed in any tissue analyzed. Although these data do not rule out cell type variation in tGolgin-1 mRNA expression, they indicate that no tissue types are enriched in cells that express particularly high or low levels of tGolgin-1 mRNA.

Because tGolgin-1 was identified using an antibody to OIP-1 expressed on oligodendrocyte precursors, we next sought to

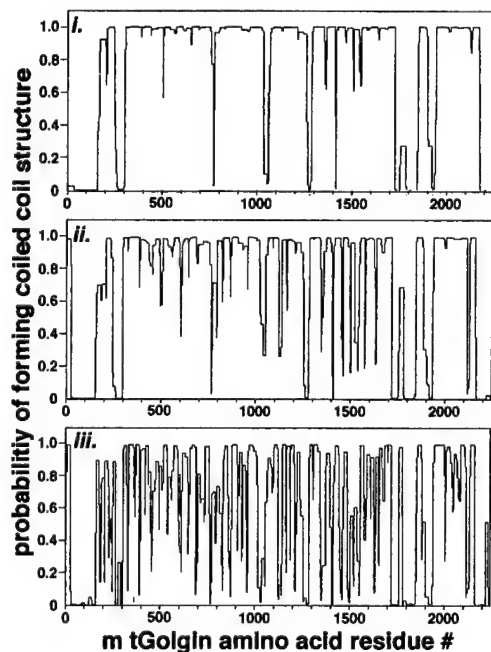
B

FIG. 2. Comparison of mouse and human tGolgin-1 amino acid sequences and coiled-coil predictions. **(A)** Deduced amino acid sequence of mouse (ms) tGolgin-1 and alignment with that of human (hu) tGolgin-1. The human tGolgin-1 sequence listed includes the N-terminal regions present in *trans*-golgi p230 and 256 kD golgin, but the C-terminal region of golgin-245 containing the region encoded by the 63bp alternatively spliced exon. Amino acid identities are darkly shaded and similarities are lightly shaded. Regions of marked homology between mouse and human sequences are indicated by thick lines above the sequence and by the letters a, b, c, and d to the right of the sequence. Potential granin motifs are boxed. **(B)** Coiled-coil predictions using the MTIDK matrix and windows of 14 (i), 21 (ii), and 28 (iii) residues without weighting of positions 1 and 4. Similar patterns were obtained with weighting of these positions and using the MTK matrix.

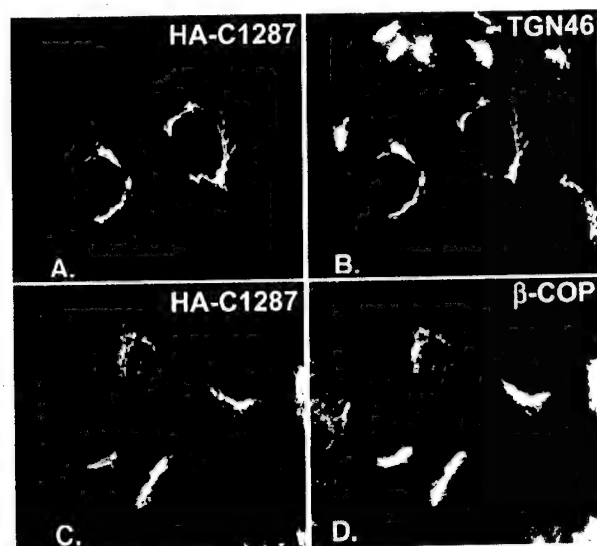


FIG. 3. The HA-tagged gene product of partial tGolgin-1 clone C91 localizes to the TGN region. (A–D) HA-C1247, representing the C-terminal 1247 residues of tGolgin-1 tagged at the N-terminus with the HA11 epitope, was expressed in HeLa cells by transient transfection. Cells were fixed and stained with antibodies to HA11 (A, C) and to either endogenous TGN46 (B) or endogenous β -COP (D) and FITC- and Rhodamine Red X-conjugated secondary antibodies. A, B and C, D show representative immunofluorescence microscopy staining patterns from identical fields of cells. Note the presence of both transfected and untransfected cells in the same fields.

determine whether endogenous tGolgin-1 mRNA was more highly expressed specifically by this cell type. Sorted populations of neonatal mouse brain cells were analyzed by semi-quantitative RT-PCR. This assay utilized limited numbers of PCR amplification cycles to assess the relative frequency of different mRNAs. First, oligodendrocyte precursors were sorted into three populations as defined by their expression of well-characterized developmental markers (see Fig. 6A and Gay *et al.*, 1997, for developmental analyses). Population 1 (O4⁺ R-Mab[−]) contains the least mature cells, which express the O4 epitope characteristic of committed oligodendrocyte precursors but not the Ranscht epitope (R-Mab) characteristic of more differentiated, less proliferative cells. Population 2 (O4⁺ R-Mab⁺) contains cells at an intermediate stage of development in which R-Mab expression has been initiated but lack expression of myelin components. Population 3 (Gal-C⁺) contains the most mature cells that express myelin components; the first two populations contained no Gal C⁺ cells, as determined by flow cytometry (unpublished data). mRNA from equal cell numbers of each population was subjected to semiquantitative PCR using various primer sets. Using primers corresponding to actin cDNA, a linear increase in accumulation of actin product was seen with increasing rounds of amplification, although both astrocytes and fibroblasts contained much higher amounts of product, most likely reflecting their larger cell size and increased mRNA levels per cell (Fig. 6A).

RT-PCR products corresponding to DM20 and PLP, two temporally regulated proteins encoded by the same gene (Macklin *et al.*, 1987), were assessed to verify the precursor stages

under study. During oligodendrocyte development, DM20 transcription precedes that of PLP (Ikenaka *et al.*, 1992; Timsit *et al.*, 1992). As predicted, the smaller DM20 product was found in population 1, whereas both the DM20 and PLP products were found in the more mature populations 2 and 3 (Fig. 6A). Astrocytes also expressed some DM20/PLP products, most likely reflecting a few mature oligodendrocytes in this unsorted cell culture. These data validate that our sorted populations were relatively pure and that the RT-PCR assay faithfully reproduced previous characterizations.

To analyze for the presence of tGolgin-1 transcripts, PCR was performed using primers corresponding to a region of the cDNA sequence present in C91 (see Fig. 1a for PCR product locations along the cDNA sequence). As can be seen in Figure 6A, a PCR product of the appropriate size was obtained from populations 2 and 3. Population 1, astrocytes, and fibroblasts contained very weak bands, which may correspond to low levels of tGolgin-1 transcripts (see below). These data indicate that tGolgin-1 mRNA is expressed most extensively by stage II precursors. This corresponds to the appropriate developmental

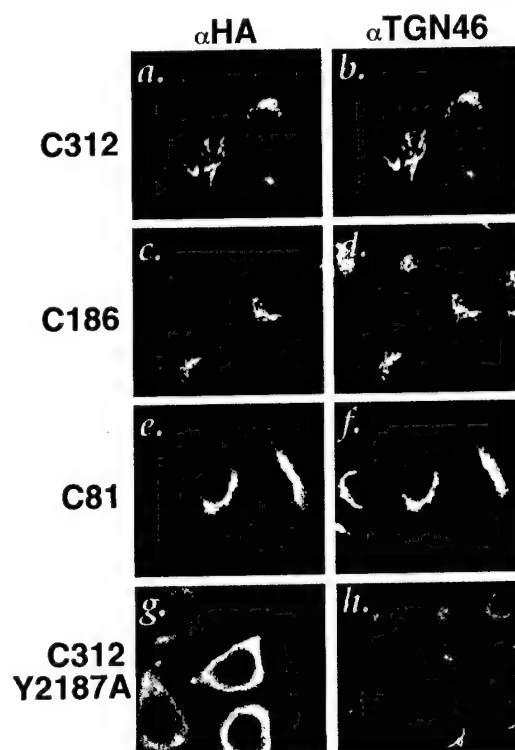


FIG. 4. GRIP domain-dependent localization of C-terminal tGolgin-1 fragments to the TGN. N-terminal HA11-epitope tagged truncations of tGolgin-1 were expressed in HeLa cells by transient transfection, and fixed cells were stained with antibodies to HA11 (a, c, e, g) and to endogenous TGN46 (b, d, f, h) and fluorophore-tagged secondary antibodies. Cells were analyzed by immunofluorescence microscopy using a conventional (a–f) or confocal scanning (g, h) microscope. The truncations comprised the C-terminal 312 (C312; a, b), 187 (C187; c, d), or 81 (C81; e, f) residues. In g, h, the cells expressed C312 with a mutation changing the conserved tyrosine at position 2187 to an alanine (C312.Y2187A).

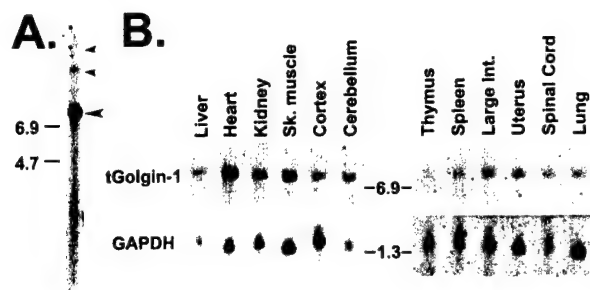


FIG. 5. Northern blot analysis of the tissue distribution of tGolgin-1 mRNA. Five micrograms of polyA⁺ RNA prepared from neonatal mouse brain (**A**) or 20 μ g of total RNA from the indicated tissues (**B**) was fractionated on agarose gels and transferred to nitrocellulose. Blots were developed with digoxigenin-conjugated probes for tGolgin-1 or GAPDH, as indicated, followed by a luminescence detection system and phosphorimaging analysis. The position of RNA markers is indicated. In **A**, the large arrowhead points to the main band estimated at 7.5 kb; this band is also highlighted in the blots in **B**. Small arrowheads in **A** point to minor RNA species only detected in longer exposures, as discussed in the text.

window for OIP-1 expression, and is the first demonstration that transcription of tGolgin-1 may be differentially regulated.

The RT-PCR analyses were extended using oligodendrocyte precursor populations sorted for expression of OIP-1 and R-Mab (Fig. 6B). OIP-1⁻R-Mab⁻ (least mature population 1), OIP-1⁺R-Mab⁺ (intermediate stage II population 2), and OIP-1⁻R-Mab⁺ (late stage II population 3) cells were subjected to identical conditions as in Figure 6A except that a second set of tGolgin-1 primers was also included for analyses. Again, only intermediate and late stage II cells generated PCR products corresponding to the tGolgin-1 sequence, and products correlated precisely with surface OIP-1 expression.

The Northern analyses described above suggested that tGolgin-1 is expressed in all tissues, and hence, in many cell types. To confirm that the RT-PCR assay could detect tGolgin-1 transcripts expressed at lower levels in cells other than oligodendrocyte precursors, two populations found negative by semi-quantitative PCR, astrocytes, and fibroblasts, were subjected to high cycle RT-PCR. At 40 cycles, weak PCR products were observed (Fig. 6C). These data support the view that the tGolgin-1 mRNA is expressed in many cells but is particularly abundant in oligodendrocyte precursors.

DISCUSSION

Identification of the murine tGolgin-1 homolog

Several groups identified and characterized a large, Golgi-localized protein as the target of autoantibodies in patients with Sjögren's Syndrome (Kooy *et al.*, 1992; Fritzler *et al.*, 1995). These groups cloned three homologous cDNAs that putatively encode proteins termed golgin-245 (Fritzler *et al.*, 1995), trans-golgi p230 (Erlach *et al.*, 1996), and 256 kD Golgin (submitted to Genbank by H. P. Seelig), all of which reacted with patient antisera, but which differed in sequence at the 5' and 3' end.

The sequences of these three proteins are nearly identical throughout their coding region; careful analysis of the sequence data indicates that they represent partial or complete clones of the same cDNA, except for the inclusion or exclusion of a 63bp exon near the coding region for the extreme C-terminus. The extreme 5' terminus of the golgin-245 sequence begins at the coding region for amino acid #130 of the trans-golgi p230 sequence, and we therefore believe that this is a 5'-truncated partial clone. The sequence of 256 kD golgin differs from that of trans-golgi p230 by the exclusion of a single G nucleotide at position 6666 within a poly-G tract; this shifts the reading frame for the predicted amino acid sequence to differ from those of trans-golgi p230 and golgin-245 near the C-terminus, and we therefore believe that this is a sequencing error. Because these proteins were localized to the trans-Golgi network and associated vesicles (Gleeson *et al.*, 1996), we refer to them collectively as tGolgin-1. Here, we identify the cDNA for the mouse homolog of tGolgin-1, and show that it is expressed in particularly great abundance in oligodendrocyte precursors.

We previously described a unique cell surface marker on stage II oligodendrocyte precursors that was referred to as OIP-1 (Gay *et al.*, 1997). Using the anti-OIP-1 mab to screen a postnatal mouse brain cDNA expression library, we isolated three overlapping cDNAs that encoded a protein with homology to the C-terminal half of human tGolgin-1. Using the mouse EST database and RT-PCR, we extended these cDNAs to span a 7530 bp region with homology to the entire human tGolgin-1 cDNA. Several data suggest that this clone represents the major or only mouse homolog of human tGolgin-1. First, the mouse tGolgin-1 clones exhibited strong homology to human tGolgin-1, and a similar splice variation of a 63 bp exon occurred at the extreme 3' end of the coding region. Second, all of the available murine EST sequences with homology to human tGolgin-1 exhibited complete or nearly complete identity with the mouse sequence. These ESTs were derived from diverse embryonic and adult cDNA libraries. Third, like its human counterpart, HA-tagged truncated forms of the mouse protein localized to the TGN in transfected cells and could be partially displaced following treatment with brefeldin A. Finally, comparable to the analyses made of human tGolgin-1 thus far, the mouse mRNA was ubiquitously expressed, albeit at much higher levels in oligodendrocyte precursors. Collectively, this evidence suggests that we have identified the only mouse homolog of human tGolgin-1.

Interestingly, the degree of similarity between the human and mouse proteins varied throughout the coding region. Regions of highest homology (>90% identical) were present at the extreme C-terminus, spanning the GRIP domain and adjacent predicted coiled-coil regions, and within the 600 amino-terminal residues. These were interspersed with regions of modest homology (60–70% identity) throughout the bulk of the protein, within regions predicted to have a coiled-coil structure. We speculate that the less conserved regions retain general structural features that are required for tGolgin-1 function, such as coiled-coil structure, whereas the more highly conserved regions likely mediate specific interactions within a tGolgin-1 oligomer or with other conserved proteins or nonprotein effectors.

It is curious that three independent cDNA clones with overlapping sequences for tGolgin-1 were identified using an anti-

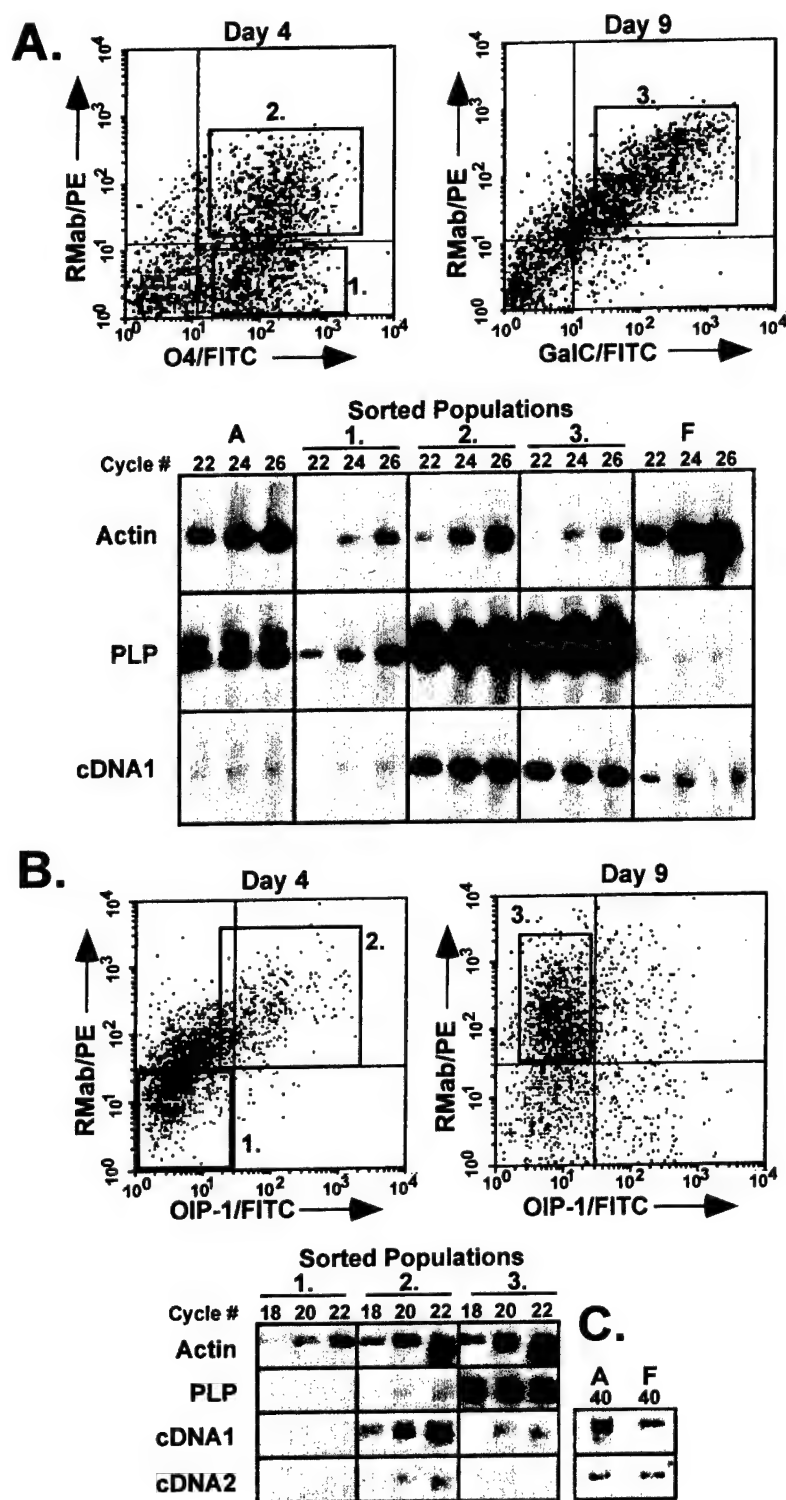


FIG. 6. tGolgin-1 mRNA is highly expressed in oligodendrocyte precursors. Semiquantitative RT-PCR (A, B) of sorted oligodendrocyte precursor populations, astrocytes, and fibroblasts and 40-cycle RT-PCR (C) of astrocytes and fibroblasts. Top panels represent flow cytometric profiles of cultured day 4 (left) or day 9 (right) mouse oligodendrocyte precursors colabeled with either anti-O4 or anti-GalC antibodies conjugated to FITC and PE-conjugated R-Mab as indicated. Sorted cells are boxed and numbered to indicate populations analyzed by RT-PCR (lower panels). Lower panels show PCR products from sorted populations, astrocytes, and fibroblasts using specific primers against designated proteins. Note that in A, PLP and cDNA1 PCR products were close to saturating levels even at the lowest cycle numbers (22, 24, 26 cycles). More representative samples, collected at lower cycle numbers (18, 20, 22 cycles), can be seen in B.

body to a cell surface marker on oligodendrocyte precursors. Although we have as yet been unable to express the full-length mouse tGolgin-1 protein in transfected cells, no cell surface expression of the clone C91 gene product could be detected with either anti-HA or anti-OIP-1 antibodies, and antibodies to human tGolgin-1 are nonreactive with the cell surface of human cell lines (unpublished data). Furthermore, except for the initial isolation from bacteriophage plaques of bacterial cultures, we were unable to demonstrate direct reactivity of the tGolgin-1 gene product with the anti-OIP-1 antibody using several assays (unpublished data). Finally, the deduced amino acid sequence contains no apparent signal sequence, consistent with the structure of a peripheral membrane protein. Nevertheless, endogenous tGolgin-1 mRNA levels were greatly upregulated in stage II oligodendrocyte precursors, compared with precursors at other developmental stages or unrelated cells such as astrocytes and fibroblasts; this is the same developmental stage at which OIP-1 surface expression is observed. We speculate that either the OIP-1 determinant represents a small amount of surface expression of tGolgin-1 in cells in which it is highly expressed, or that high levels of tGolgin-1 protein are required to generate a determinant on a distinct molecule that can be recognized by the anti-OIP-1 mab (see below). Surface expression of a normally cytoplasmic protein would be unusual, but not unprecedented (Cleves and Kelly, 1996). Indeed, cellular myosin II, a protein with some homology to tGolgin-1 that also participates in vesicular transport from the TGN (Musch *et al.*, 1997; Stow *et al.*, 1998), has been reported to associate tightly with the neuronal plasma membrane (Li *et al.*, 1994) and to localize to the cell surface (Michelis *et al.*, 1994; Yanase *et al.*, 1997).

A possible function for tGolgin-1 in the vesicular trafficking of glycolipids or proteolipids?

It has been postulated that components of the vesicular transport machinery are upregulated to accommodate myelination. Indeed, Rab3a and Rab3c, GTP-binding proteins associated with regulated secretion in neurons and other specialized cell types, have been shown to be upregulated during the late development and maturation of oligodendrocytes (Madison *et al.*, 1996). Oligodendrocytes have been shown to possess features of polarized sorting (de Vries *et al.*, 1998), and express a unique Rab protein, Rab22b, which is thought to be involved in traffic between the TGN and endosomes (Rodriguez-Gabin *et al.*, 2001). We found elevated tGolgin-1 mRNA levels at a point in development slightly earlier than that in which myelin deposits accumulate, that immediately preceding and coincident with high surface expression of R-Mab antigen. These results suggest that expression of tGolgin-1 can be upregulated to accommodate specific vesicular transport functions in oligodendrocyte precursors, such as an increased cargo load or specialized cargo. A role in the transport of specialized cargo is supported by the finding that human tGolgin-1 and another TGN-associated peripheral membrane protein, myosin II, are present on separate vesicles associated with the TGN (Gleeson *et al.*, 1996; Brown *et al.*, 2001).

What might this specialized cargo be? Given the unique role of glycosphingolipids in oligodendrocyte biology, we speculate that tGolgin-1 may be involved in transport or synthesis of gly-

colipids. Glycolipids, including sulfatides such as the R-Mab antigen, are highly enriched in stage II oligodendrocyte precursors (Bansal *et al.*, 1989; Bansal and Pfeiffer, 1992) at the same stage in which we find high levels of tGolgin-1 mRNA. Glycolipids are manufactured and assembled into myelin at the TGN (Simons *et al.*, 2000) and progress to the cell surface through vesicular transport (Burkart *et al.*, 1982; Gow *et al.*, 1994). Glycosphingolipids play a general role in regulating Golgi export (Sprong *et al.*, 2001b), protein sorting processes at the TGN (van Meer, 1998; Sprong *et al.*, 2001a), and retrograde transport from the plasma membrane to the TGN (Falguières *et al.*, 2001; Johannes and Goud, 1998). Moreover, the differentiation of oligodendrocytes has been shown to be associated with distinct glycolipid sorting processes (Watanabe *et al.*, 1999), and maintenance of oligodendrocyte processes requires glycolipid cycling between the plasma membrane and the TGN (Benjamins and Nedelkoska, 1994). We speculate that tGolgin-1 may be involved in either the transport of glycolipids or sulfatides such as the R-Mab antigen (Bansal *et al.*, 1989; Bansal and Pfeiffer, 1992), or in the localization of resident TGN enzymes that facilitate glycolipid or sulfatide synthesis. Perhaps the anti-OIP-1 antibody recognizes a glycolipid that binds to tGolgin-1 and for which cell surface expression or recycling is facilitated by tGolgin-1.

ACKNOWLEDGMENTS

We thank Diane Murphy for her help with confocal microscopy, Avinash Bhandoola for guidance in preparing and screening the cDNA library, and Dawn Harper for assistance in preparation of some of the constructs. D.G. was supported by the National Science Foundation grant NSF IBN-9631764, and grants from the National Multiple Sclerosis Foundation and Lucille P. Markey Charitable Trust. M.I.G. was partially supported by grant PO1 NS11037 from the National Institutes of Health. M.S.M., D.A.C., A.Y., and B.M.B. were supported by American Cancer Society Grants RPG-00-238-01-CSM and RPG-97-003-01-BE, and A.Y. was partially supported by a fellowship from the United States Department of the Army.

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Received for publication March 13, 2002; accepted March 15, 2002.

Golgi Recruitment of GRIP Domain Proteins by Arf-like GTPase 1 Is Regulated by Arf-like GTPase 3

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Summary

Golgins are Golgi-localized proteins present in all molecularly characterized eukaryotes that function in Golgi transport and maintenance of Golgi structure. Some peripheral membrane Golgins, including the yeast Imh1 protein, contain the recently described GRIP domain that can independently mediate Golgi localization by an unknown mechanism [1–3]. To identify candidate Golgi receptors for GRIP domain proteins, a collection of *Saccharomyces cerevisiae* deletion mutants was visually screened by using yeast, mouse, and human GFP-GRIP domain fusion proteins for defects in Golgi localization. GFP-GRIP reporters were localized to the cytosol in cells lacking either of two ARF-like (ARL) GTPases, Arl1p and Arl3p. In vitro binding experiments demonstrated that activated Arl1p-GTP binds specifically and directly to the Imh1p GRIP domain. Arl1p colocalized with Imh1p-GRIP at the Golgi, and Golgi localization of Arl1p was regulated by the GTPase cycle of Arl3p. These results suggest a cascade in which the GTPase cycle of Arl3p regulates Golgi localization of Arl1p, which in turn binds to the GRIP domain of Imh1p and recruits it to the Golgi. The similar requirements for localization of GRIP domains from yeast, mouse, and human when expressed in yeast, and the presence of Arl1p and Arl3p homologs in these species, suggest that this is an evolutionarily conserved mechanism.

Results and Discussion

When expressed in mammalian tissue culture cells, isolated GRIP domains from yeast and mammalian proteins localize to the Golgi, and high-level expression of isolated GRIP domains displaces endogenous GRIP domain-containing proteins from the Golgi [1, 3]. These data suggest that a limiting, evolutionarily conserved receptor at the Golgi is responsible for recruitment of GRIP domain proteins. The yeast genome encodes a single protein, Imh1p, with a GRIP domain, and a GFP-Imh1p GRIP domain fusion protein localizes to punctate organelles that likely correspond to the Golgi [1]. We expressed the Imh1p GRIP domain as a fusion to a variant red fluorescent protein (RFP), DsRedT.4 [4], in

cells coexpressing Sec7p-GFP, a pan Golgi marker protein [5], and Chs3p-GFP, which is localized predominantly to the late Golgi/post-Golgi endosome [6]. Nearly perfect colocalization was observed with Chs3p-GFP, and substantial colocalization was observed with Sec7p-GFP (Figure 1). Identical results were obtained with full-length Imh1p, and deletion of the GRIP domain from Imh1p resulted in cytosolic localization (data not shown), indicating that its GRIP domain mediates localization of the full-length protein, as is the case for human p230/tGolgin-1 [7]. In control experiments, no colocalization was observed in yeast expressing a GFP-FYVE domain protein (derived from human EEA1) that labels prevacuolar endosomes (data not shown) [8]. These results definitively demonstrate that the Imh1p GRIP domain localizes to the yeast Golgi and preferentially to late Golgi compartments.

To determine if the yeast Golgi contains a receptor that can be recognized by mammalian GRIP domains, we expressed GFP fusions to the mouse tGolgin-1 [9] and human Golgin-97 [10] GRIP domains in wild-type yeast cells. Both GRIP domains localized to punctate organelles (Figure 2), indicating that whatever element of the Golgi serves to recruit GRIP domain proteins is conserved between yeast and mammals. We hypothesized that since *IMH1* is not an essential gene, the Golgi receptor that recruits Imh1p might also be encoded by a nonessential gene(s) or activity. Thus, to identify a candidate Golgi-localized receptor for GRIP domain proteins, we expressed the GFP-GRIP domain of yeast Imh1p in approximately 250 yeast strains with deletions of genes encoding nonessential, known, or predicted Golgi-localized proteins or proteins known to regulate traffic to, through, or out of the Golgi, and we screened these strains by fluorescence microscopy for loss of Golgi localization. In two strains, *arl1Δ* and *arl3Δ*, the GFP reporter was no longer localized to the Golgi and appeared cytosolic (Figure 2). Localization of mouse tGolgin-1 and human Golgin-97 GFP-GRIP domains in these mutant strains was similarly affected (Figure 2). Arl1p and Arl3p are divergent members of the ARF family of GTPases, referred to as ARF-like or ARL GTPases, and they are highly conserved with the human ARL1 and ARF-related protein (ARP) GTPases, respectively [11].

Yeast *arl1Δ*, *arl3Δ*, and *imh1Δ* mutants have minor defects in sorting proteins to the lysosome-like vacuole; these defects suggest a role for these proteins in biosynthetic protein sorting in the late Golgi or in endosomes [12–16]. Moreover, human ARL1 has been localized to the Golgi of transfected cells and has been implicated in regulating Golgi structure and Golgi protein sorting [17, 18]. These findings, and the general observation that localization of many peripheral membrane proteins is often regulated by a Ras-related GTPase [19], led us to explore the possibility that Arl1p or Arl3p might serve as a receptor for GRIP domains on the Golgi. To test this, we purified recombinant Arl1p and Arl3p and assayed for binding to a GST fusion protein containing the yeast Imh1p GRIP domain. As shown in Figure 3, the constitu-

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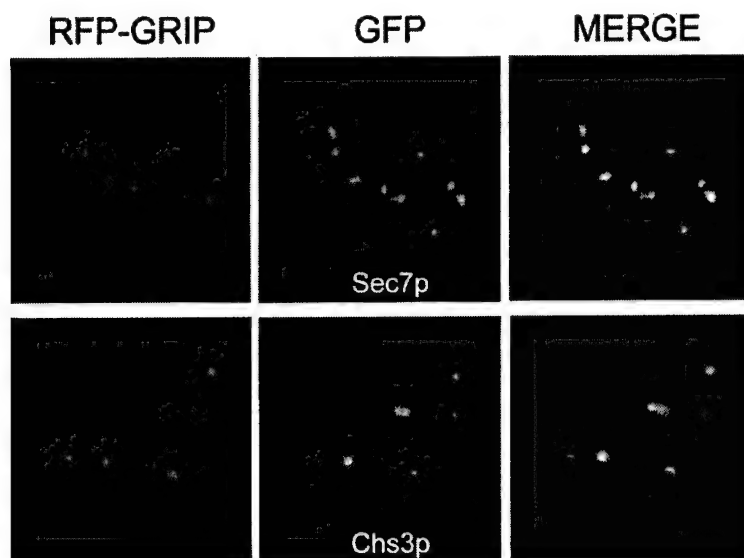


Figure 1. Imh1p GRIP Domain Localizes to Golgi Compartments

A red fluorescent protein (RFP)-Imh1p-GRIP fusion (encoding amino acids 735–912) was expressed in wild-type strains (BY4741 or BY4742) expressing Sec7-GFP or Chs3p-GFP, which both localize to Golgi compartments. Colocalization can be observed as yellow in the panels on the right.

tively active (GTPase-deficient, Q72L) form, but not the GTP binding-defective (GDP-locked, T32N) form, of Arl1p bound to GST-GRIP. The binding was specific, as no binding to Arl3p, either in the GTP- or GDP-locked forms (Q78L, T31N), or by recombinant yeast Arl3/Arl2p or the Rab GTPases, Ypt31p, Ypt7p, or Vps21p, was observed (data not shown). Furthermore, coincubation of Arl3p had no effect on Arl1p binding. These results indicate that the Imh1p GRIP domain binds directly and specifically to Arl1p-GTP. Because only a small proportion of Arl1p (approximately 5% of input) bound to the Imh1p GST-GRIP domain fusion protein, it is possible that myristoylation of Arl1p influences this interaction, or that within the cell, additional factors, such as other Golgi-localized proteins and/or lipids, contribute to Imh1p Golgi localization.

If Arl1p is a Golgi receptor for GRIP domain proteins, then the Imh1p GRIP domain and Arl1p should colocalize at the Golgi. To test this, we tagged the chromosomal *ARL1* locus with GFP so that it served as the only source of Arl1p and transformed this strain with the RFP-Imh1p-GRIP plasmid. Arl1p-GFP localized to punctate organelles and colocalized perfectly with RFP-Imh1p-GRIP, indicating that Arl1p is indeed localized to the Golgi (Figure 4). In contrast to the results with Arl1p, in cells expressing Arl3p-GFP, very few cells exhibited a punctate signal and very weak cytoplasmic fluorescence was observed (see Figure S1 in the Supplementary Material available with this article online). Because membrane targeting of most characterized ARF GTPases is regulated by their GTPase cycle, we examined localization of GFP-tagged, constitutively active Arl3p(Q78L) and

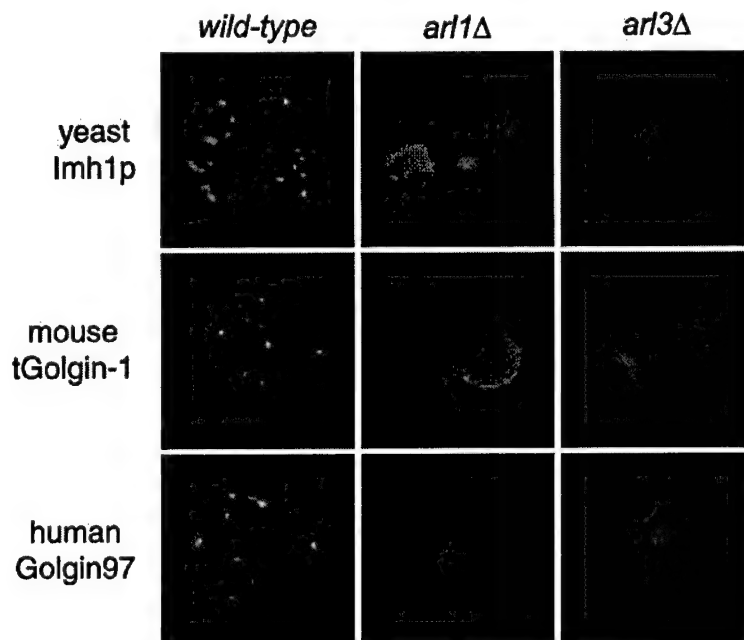


Figure 2. Golgi Localization of Yeast Imh1p, Mouse tGolgin-1, and Human Golgin-97 GRIP Domains Is Disrupted in *arl1Δ* and *arl3Δ* Mutants

GFP fusions to the Imh1p GRIP domain, the mouse tGolgin-1 GRIP domain (amino acids 2053–2238), or the human Golgin-97 GRIP domain (amino acids 588–767) were expressed in wild-type, *arl1Δ*, and *arl3Δ* strains and were photographed.

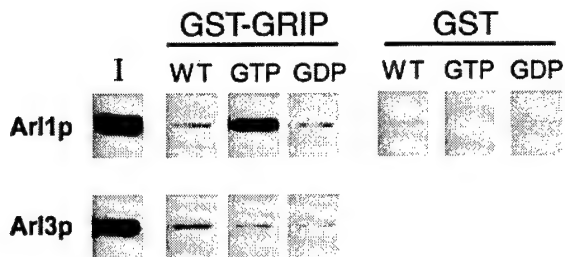


Figure 3. Activated Arl1p Binds the GST-lmh1p GRIP Domain

A fusion protein consisting of GST coupled to the lmh1p GRIP domain, or GST as a control, was captured on GSH Sepharose and mixed with purified, recombinant wild-type ("WT"), GTPase-deficient ("GTP"), or GTP binding-defective ("GDP") Arl1p or Arl3p. After incubation, the beads were washed twice, and bound material was visualized by immunoblotting with antibodies to epitope-tagged Arl GTPases. Approximately 10% of the added input of each Arl GTPase is shown in the "I" lane (only the activated ["GTP"] Arl input lanes are shown).

inactive Arl3p(T31N) mutants expressed in *arl3Δ* cells from a single copy vector. A faint punctate signal was observed for constitutively active Arl3p(Q78L)-GFP (Figure 4), but Arl3p(T31N)-GFP was apparently localized to the cytosol (Figure S1). These observations suggest that Arl3p is localized to the Golgi in a nucleotide-dependent manner, although the low Arl3p-GFP signal precluded definitive colocalization with Golgi markers. Interestingly, Arl3p and its human ortholog ARP lack the consensus Glycine that is myristoylated in all characterized ARF GTPases, and this may explain in part why Arl3p is poorly localized to the Golgi at steady state.

The observations that GRIP domains are mislocalized in the *arl3Δ* strain, but that Arl3p does not appear to bind the lmh1p GRIP domain, led us to hypothesize that Arl3p might regulate localization of Arl1p. To test this hypothesis, we examined localization of GFP-tagged

Arl1p in *arl3Δ* mutant cells and found that it was localized to the cytosol in *arl3Δ* cells, indicating that Arl3p functions to promote stable association of Arl1p with the Golgi (Figure 4). To determine if the nucleotide cycle of Arl3p regulates Arl1p-dependent Golgi localization of GRIP domains, we next examined localization of GFP-lmh1p-GRIP and Arl1p-GFP in *arl3Δ* mutant cells transformed with single copy plasmids expressing Arl3p(T31N) or Arl3p(Q78L) as the sole sources of Arl3p. As can be seen in Figure 4 and in Figure S1, both GFP-lmh1p-GRIP and Arl1-GFP were localized to the Golgi when activated Arl3p(Q78L) was present, but not when Arl3p(T31N) was present. Thus, Arl3p indirectly affects Arl1p-dependent localization of GFP-GRIPs because, in *arl3Δ* cells, Golgi localization of the GRIP domain receptor, Arl1p-GTP, is abrogated.

These results lead us to propose a model in which the nucleotide cycle of Arl3p regulates Golgi localization of Arl1p, which, when activated by nucleotide exchange, recruits lmh1p to the Golgi via its GRIP domain. Orthologs of yeast Arl3p, Arl1p, and lmh1p are present in other eukaryotes, and because localization of mammalian GRIP domains in yeast requires the same proteins as yeast lmh1p, the *ARL3/ARL1/IMH1* pathway appears to be conserved throughout eukaryotic evolution. Other components of this pathway are likely to include guanine nucleotide exchange factors (GEFs) and GTPase activating factors (GAPs) that regulate the nucleotide cycles of Arl3p and Arl1p. Mon2p (also called Ysl2p) was reported to be related to Sec7 family ARF GEFs, and high-level expression of *ARL1*, but not *ARL3*, was found to suppress the growth defect of a *mon2Δ* deletion mutant [20], raising the possibility that Mon2p may be a GEF for Arl1p. However, localization of Arl1p-GFP and GFP-lmh1p-GRIP is not substantially affected in a *mon2Δ* mutant (data not shown), although genetic evidence does implicate Mon2p as a component of the *ARL3/ARL1/IMH1* pathway. Analysis of Golgi protein sorting

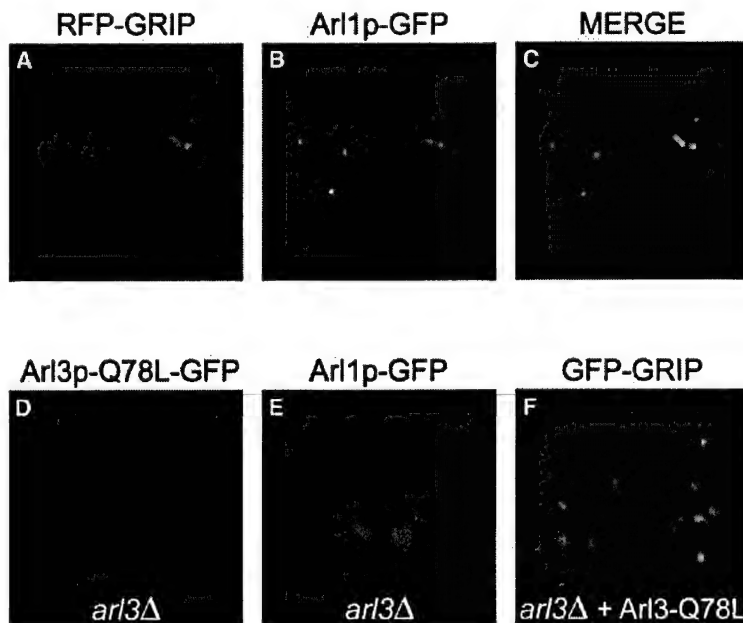


Figure 4. The GTPase Cycle of Arl3p Regulates Golgi Localization of Arl1p and GFP-lmh1p-GRIP

(A-C) RFP-lmh1p-GRIP and Arl1p-GFP were visualized by fluorescence microscopy individually (A and B), and then the two micrographs were overlaid (C).

(D) To visualize Arl3p within the cell, the GFP-tagged wild-type, inactive Arl3p(T31N) or constitutively active Arl3p(Q78L) were expressed from single copy vectors in the *arl3Δ* strain and were photographed (see Figure S1). (E) Localization of Arl1p in *arl3Δ* mutant cells was determined by expressing GFP-tagged wild-type Arl1p from a single copy vector that had been transformed into the *arl3Δ* strain. To determine if the GTPase cycle of Arl3p regulates Arl1p-dependent Golgi localization of the lmh1p GRIP domain, the *arl3Δ* strain was cotransformed with single copy vectors expressing wild-type or mutant *arl3* genes and the GFP-lmh1p-GRIP plasmid.

(F) Golgi localization of GFP-lmh1p-GRIP was observed in wild-type and Arl3p(Q78L) cells, but not in Arl3p(T31N) cells (Figure S1).

in *imh1*, *arl1*, and *arl3* mutants, and identification of genetic interactions between mutations in *imh1* and mutations affecting the Ypt6 Rab GTPase, suggest that it may serve in the retention and/or retrieval of proteins to the late Golgi [16, 20, 21, 22]. We have found that *arl1Δ*, *arl3Δ*, and *mon2Δ* alleles lead to synthetic lethality when combined with a deletion of *ypt6* (Figure S2), as has been reported for an *imh1Δ ypt6Δ* double mutant [23]; this finding is consistent with these genes functioning together in a genetic pathway that is essential in the absence of Ypt6p.

Our findings are consistent with a cascade of ARF-like GTPases that function in a sequential manner to regulate protein localization in the Golgi. A cascade of sequentially acting yeast Rab GTPases that regulates vectorial transport of cargo to and through the Golgi has recently been described [24, 25], and it may be that an Arl GTPase cascade functions in an analogous manner to regulate recycling of proteins to the Golgi from endosomal compartments. This work establishes functions for the two yeast ARF-like GTPases and should open the door to a better understanding of the roles of this family of enzymes in regulating Golgi function.

Supplementary Material

Supplementary Material including a description of the Experimental Procedures used to construct and visualize the GFP- and RFP-tagged proteins and to perform the GRIP domain binding experiments is available at <http://images.cellpress.com/supmat/supmatin.htm>.

Acknowledgments

This work is supported by grants from the National Institutes of Health to C.G.B. (GM61221) and from the American Cancer Society to M.S.M. (RPG-00-238-01-CSM). We thank Efe Bi, Mark Lemmon, and Margaret Chou for helpful discussions and critical reading of the manuscript, Ben Glick and Randy Schekman for sharing reagents, and Charlie Boone, Amy Tong, and Sean Munro for sharing information before publication.

Received: November 25, 2002

Revised: December 20, 2002

Accepted: December 20, 2002

Published: March 4, 2003

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A role for GRIP domain proteins and/or their ligands in structure and function of the *trans* Golgi network

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Running title:

GRIP domain proteins and TGN homeostasis

Key words:

golgin-245/p230, golgin-97, TGN46, endosomes, VSV-G

SUMMARY

tGolgin-1 (golgin-245, trans golgi p230) and golgin-97 are members of a family of peripheral membrane proteins of unknown function that localize to the *trans* Golgi network (TGN) through a conserved C-terminal GRIP domain. Here, we probe for GRIP protein function by assessing the consequences of overexpressing isolated GRIP domains. By semi-quantitative immunofluorescence microscopy, high level expression of epitope-tagged, GRIP domain-containing fragments of tGolgin-1 or golgin-97 specifically altered the characteristic pericentriolar distribution of TGN integral membrane and coat components. Concomitantly, vesicular transport from the TGN to the plasma membrane and furin-dependent cleavage of substrate proteins in the TGN were inhibited. Mutagenesis of a conserved tyrosine in the tGolgin-1 GRIP domain abolished these effects. GRIP domain overexpression had little effect on the distribution of most Golgi stack resident proteins and no effect on markers of other organelles. Electron microscopy analyses of GRIP domain overexpressing cells revealed distended perinuclear vacuoles and a proliferation of multivesicular late endosomes to which the TGN resident protein TGN46 was largely mislocalized. These studies, the first to address the function of GRIP domain-containing proteins in higher eukaryotes, suggest that some or all of these proteins and/or their ligands function in maintaining the integrity of the TGN by regulating resident protein localization.

7715 words; summary, 203 words

INTRODUCTION

The *trans* Golgi network (TGN) is a series of interconnected tubules and vesicles at the *trans* face of the Golgi stack that functions in the processing and sorting of glycoproteins and glycolipids at the interface of the biosynthetic and endosomal pathways (Griffiths and Simons, 1986; Traub and Kornfeld, 1997). TGN structure is dynamic, subject to constant influx and efflux of membrane from and to both secretory and endosomal compartments. Such dynamics require efficient membrane recycling to maintain a constant steady state composition of lipids and proteins. Hence, TGN resident integral membrane proteins, including glycosyl modifying enzymes (Geuze and Morre, 1991), proprotein processing enzymes (Seidah and Chretien, 1997; Varlamov and Fricker, 1998), SNARE proteins (Lewis et al., 2000; Mallard et al., 2002; Siniossoglou and Pelham, 2001), and putative cargo binding proteins such as TGN38/ TGN46 (Banting and Ponnambalam, 1997), maintain a steady state accumulation within the TGN by active retention and recycling (Bryant and Stevens, 1997; Ghosh et al., 1998; Mallet and Maxfield, 1999; Ponnambalam et al., 1994). The best characterized recycling pathways involve retrieval of resident proteins and glycolipids from endosomes (Molloy et al., 1999; Rohn et al., 2000). The molecular mechanisms that regulate both the efflux of membrane to the cell surface and the retrieval from endosomes are only beginning to be understood, and include sorting signals on cargo proteins and specific cytoplasmic components to effect cargo movement (Mallard et al., 2002; Rohn et al., 2000). Among these components are likely to exist as yet unidentified tethering factors (Lowe et al., 1998; Pfeffer, 1999).

In an effort to elucidate effectors regulating TGN biogenesis, we have focused on a group of large peripheral membrane proteins of unknown function characterized by an extensive predicted coiled-coil structure and a conserved C-terminal GRIP (Golgin-97, RanBP2 α , Imh1p and trans golgi p230) domain (Barr, 1999; Kjer-Nielsen et al., 1999a; Kjer-Nielsen et al., 1999b; Munro and Nichols, 1999). The four mammalian GRIP domain-containing proteins (GRIP proteins), tGolgin-1 (Cowan et al., 2002; also known as: trans golgi p230, Erlich et al, 1996; golgin-245, Fritzler et al.

1995; and 256kDa golgin, H.P. Seelig, Genbank accession # X82834), golgin-97, GCC88 and GCC185, localize to the TGN and associated vesicles via the GRIP domain (Brown et al., 2001; Gleeson et al., 1996; Luke et al., 2003). The single yeast GRIP protein, Imh1p, similarly localizes to the late Golgi (Panic et al., 2003; Setty et al., 2003). No function has yet been assigned to any GRIP protein, but indirect evidence supports a role in vesicular traffic at the TGN. Several GRIP domains bind to the small Arf-like GTPase, Arl1 (Lu et al., 2001; Panic et al., 2003; Setty et al., 2003; Van Valkenburgh et al., 2001), which itself may regulate Golgi and/or TGN structure (Lu et al., 2001; Van Valkenburgh et al., 2001; Panic et al., 2003). tGolgin-1 is associated with a class of vesicles released from Golgi membrane fractions in an in vitro budding assay (Brown et al., 2001; Gleeson et al., 1996). Finally, *IMH1* displays both multicopy suppression and synthetic lethality with mutations in genes encoding the yeast Rab6 homologue, *YPT6*, and its nucleotide exchange factor, *RIC1* (Li and Warner, 1996; Siniosoglou et al., 2000; Tsukada and Gallwitz, 1996; Tsukada et al., 1999), which themselves are implicated in endosome to TGN recycling (Bensen et al., 2001; Siniosoglou and Pelham, 2001; Tsukada et al., 1999). Given these data, and their structural similarities to known tethering proteins (Pfeffer, 1999), we hypothesized that mammalian GRIP proteins function in regulating protein recycling from endosomes to the TGN.

Overexpression of isolated GRIP domains by transfection competitively displaces endogenous GRIP proteins from Golgi membranes (Kjer-Nielsen et al., 1999a; Kjer-Nielsen et al., 1999b), presumably by competition for a limiting GRIP domain binding site. Such displacement would be expected to interfere with the function of endogenous GRIP proteins. We show here that in cells in which GRIP domain-containing fragments from tGolgin-1 or golgin-97 are overexpressed, the structure, resident protein localization, and function of the TGN are largely disrupted. The results suggest that GRIP proteins or their ligands function in the maintenance of TGN integrity, likely through regulating TGN membrane protein localization.

MATERIALS AND METHODS

Plasmids. cDNA clones encoding mouse tGolgin-1 and plasmids encoding N-terminally HA11 epitope-tagged truncated forms encoding the C-terminal 312, 186, 81 or 50 amino acids of tGolgin-1 (C312, C186, C81, C50) and a point mutant of C312, C312(Y2187A) (in which the codon for the critical GRIP domain tyrosine residue was altered to that for an alanine residue) in the mammalian expression vector pCDM8.1 (Bonifacino et al., 1990) are described elsewhere (Cowan et al., 2002). T7 epitope-tagged C312 in pCDM8.1 was prepared by two-step polymerase chain reaction (Higuchi et al., 1988) using primers encoding the epitope tag and a Kozak consensus start site. A fragment encoding the C-terminal 179 amino acids of golgin-97 (G97-C179) was amplified by reverse transcriptase coupled PCR (RT-PCR) from RNA isolated from a human melanoma, MNT-1, and subcloned into pCDM8.1-HA11. Sequences of all PCR-derived inserts and of junctions of subcloned fragments were verified by automated dideoxy sequencing. Details of the sequence of PCR primers used for plasmid construction will be provided upon request. Expression vectors for Tac (Leonard et al., 1984) or for Tac chimeric proteins with the cytoplasmic domains of TGN38 (Humphrey et al., 1993), furin (Voorhees et al., 1995), Lamp1 (Marks et al., 1996), or the dileucine-based lysosomal sorting signal of CD3 γ (Letourneur and Klausner, 1992; Marks et al., 1996) in pCDM8.1, for Pmel17 in pCI (Berson et al., 2001), for furin with a C-terminal HA epitope tag in pXS (Bosshart et al., 1994), and for the ts045 variant of the vesicular stomatitis virus glycoprotein conjugated to enhanced green fluorescent protein (Presley et al., 1997) have been described.

Cell culture and transfections. HeLa cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Cells were transfected using calcium phosphate precipitation as described (Marks et al., 1996). For most experiments, cells grown on coverslips in 6 well dishes were transfected with 7 μ g of total DNA; in cases where moderate expression levels were desired, 50-100 ng of the desired expression construct were used. Except

where noted, cells were analyzed 2-3 days following transfection. Stable transfectants of the CHO cell variant TRVb-1 expressing Tac chimeric proteins TGG and TTF (Ghosh et al., 1998; Mallet and Maxfield, 1999), provided by Dr. F. R. Maxfield (Weil Medical College of Cornell Univ., New York, NY), were transiently transfected using Superfect (Qiagen, Valencia, CA) or GenePORTER (Gene Therapy Systems, San Diego, CA) according to the manufacturers' instructions.

Antibodies. The following antibodies were used, with sources listed parenthetically. HA11 epitope (mab 16D12, Covance Research Products, Richmond, CA; mabs 12CA5 and 3F10, Roche Molecular Biochemicals, Indianapolis, IN); TGN46 (rabbit anti-TGN46, Prescott et al., 1997; sheep anti-TGN46, Serotec, Oxford, UK); cation independent mannose 6-phosphate receptor (mab α MPR300, Affinity BioReagents, Golden, CO); Tac (Santini et al., 1998); golgin-97 (rb α golgin-97 and mab α golgin-97, E. K. L. Chan, Scripps Res. Inst., La Jolla, CA); giantin (Linstedt and Hauri, 1993); Rab6 (C-19 rb α Rab6, Santa Cruz Biotechnology, Santa Cruz, CA); ERGIC-53 (Schweizer et al., 1988); mannosidase II (Moremen and Touster, 1985); galactosyltransferase (Berger and Hesford, 1985); MG160 (Gonatas et al., 1989); AP1 (Ahle et al., 1988); Lamp1 (Carlsson et al., 1988; Mane et al., 1989); Lamp2 (Mane et al., 1989); CD63 (mab α CD63, Beckman Coulter, Fullerton, CA); transferrin receptor (mab B3/25, Roche Molecular Biochemicals); T7 epitope (mAb from Novagen, Madison, WI). Mabs to EEA1, human tGolgin-1 (p230), GM130, p115, and syntaxin 6 were from Becton Dickinson/Transduction Laboratories (San Diego, CA). Secondary antibodies to mouse, rabbit, sheep or rat IgG conjugated to rhodamine red X (RRX), fluorescein isothiocyanate (FITC), aminomethylcoumarin acetate (AMCA), or gold (6nm and 12nm) were from Jackson ImmunoResearch (West Grove, PA). FITC- and Texas Red-conjugated secondary antibodies to mouse IgG isotypes were from Southern Biotechnology (Birmingham, AL).

Immunofluorescence microscopy. Cells were fixed with 2% formaldehyde and stained as described (Marks et al., 1995). Where indicated, cells were treated with 1 mg/ml leupeptin or 10-50

$\mu\text{g/ml}$ cycloheximide (CHX) prior to fixation. For transferrin uptake, cells on coverslips were incubated with FITC-transferrin (from Sigma; $20 \mu\text{g/ml}$) in serum-free medium containing 0.5% bovine serum albumin for 15-20 min, then rinsed in warm PBS prior to fixation. Cells were analyzed on a Leica (Bannockburn, IL) DM IRBE microscope using a Hamamatsu (Hamamatsu, Japan) Orca digital camera and Improvision (Lexington, MA) OpenLab software. Semiquantitative analyses were done with the Measurements module using raw images taken at constant exposure times pre-determined to be sub-saturating for the brightest samples. Outlines were drawn around individual cells, and total fluorescence from each cell was determined by multiplying the total number of pixels within the outline by the average value per pixel (after subtracting a background value per pixel, taken from an area within the photographed field in which there were no cells). Images shown were obtained using the Volume Deconvolution module from a series of raw images in different z-axis planes.

Metabolic pulse/chase and immunoprecipitation. Cells transfected with C312, C312(Y2187A), or control vector together with pCI-Pmel17 were metabolically labeled for 30 min with ^{35}S -methionine/ cysteine and chased for various periods of time, cell lysates in Triton X-100 were immunoprecipitated with anti-Pmel17, immunoprecipitates were fractionated by SDS-PAGE, and gels were analyzed by phosphorimaging as described (Berson et al., 2001).

Electron microscopy analyses. HeLa cells cotransfected with C312 or C312(Y2187A) ($42 \mu\text{g}/10\text{cm}$ dish) and pCDM8.1-Tac ($4.2 \mu\text{g}$) (Marks et al., 1995) were harvested in PBS/ 5mM EDTA, washed, and stained with anti-Tac antibodies conjugated to phycoerythrin (Beckman Coulter, Fullerton, CA). Fluorescent labeled cells were harvested using a FACStar Plus cell sorter. Sorted cells were fixed as described previously (McCaffery and Farquhar, 1995) after a 15 or 30 min incubation at 37°C with 5 mg/ml horseradish peroxidase (Sigma). For conventional electron microscopy (EM), cells were fixed in 100mM cacodylate buffer, pH 7.4, containing 3%

formaldehyde, 1.5% glutaraldehyde, and 2.5% sucrose for 1 hr, washed, and osmicated at 4°C in Palade's fixative containing 1%OsO₄. Cells were then washed, treated with tannic acid, stained with uranyl acetate, dehydrated through a graded series of ethanol, and embedded in epon. 80nm sections were cut on a LEICA UCT ultramicrotome and analyzed on a Philips 420 TEM at 80kV. For immunogold labeling of ultrathin cryosections, cells were fixed in PBS containing 4% paraformaldehyde for 1 hr, washed and harvested. Cell pellets were cryo-protected in 2.3M sucrose containing 20% polyvinyl pyrrolidone, mounted on aluminum cryo-pins, and frozen in liquid N₂. Ultrathin cryosections were then cut on a Reichert UCT ultramicrotome equipped with an FCS cryo-stage, and sections were collected onto 300 mesh, formvar/carbon coated nickel grids. Grids were washed, blocked in 10% FCS, and incubated overnight with primary antibodies (10µg/ml). After washing, grids were incubated with gold-conjugated secondary antibodies for 2hr, washed, and embedded in a mixture containing 3.2% polyvinyl alcohol (10K m.w.), 0.2% methyl cellulose (400 centiposes), and 0.2% uranyl acetate. Sections were analyzed on a Philips EM 410 transmission electron microscope.

RESULTS

Disruption of TGN protein localization by overexpression of GRIP domains. In earlier work, HA-epitope-tagged, truncated forms of mouse tGolgin-1 containing the C-terminal 1247, 312, 186 or 81 amino acids, encompassing the GRIP domain, were efficiently localized to the Golgi when expressed at low levels by transfection in HeLa cells or other cell lines (Cowan et al., 2002). To probe GRIP protein function, we used immunofluorescence microscopy (IFM) to analyze whether high level expression of GRIP domain-containing fragments in transiently transfected HeLa cells affected the distribution of TGN resident proteins. HA-tagged C312 (corresponding to the C-terminal 312 amino acids of mouse tGolgin-1) was used in most experiments because of its enhanced expression and stability relative to the other truncated products, but similar results were observed using all GRIP domain-containing tGolgin-1 fragments. As the expression levels of C312 increased, pericentriolar anti-HA staining gave way to a diffuse staining pattern throughout the cytoplasm (**Fig. 1b, c**). We defined three qualitative fluorescence patterns: level I, a narrow ribbon-like structure consistent with Golgi localization; level II, a more diffuse paranuclear/pericentriolar structure with additional cytoplasmic staining; and level III, an intense cytoplasmic staining that may mask underlying structure. The C312 distribution in these cells was often reticular, perhaps reflecting association with ER membranes or cytoskeletal elements. Semiquantitative analysis of expression levels showed a correlation between total fluorescence intensity and the staining pattern observed (**Fig. 1d**). Consistent with previous reports (Kjer-Nielsen et al., 1999a; Kjer-Nielsen et al., 1999b), cells with level II or III staining patterns displayed reduced or eliminated pericentriolar staining for endogenous tGolgin-1 and golgin-97 (see **Fig. 3**), likely reflecting competition for a limited GRIP domain binding site at the TGN (Barr, 1999; Kjer-Nielsen et al., 1999b).

Surprisingly, the localization of several TGN resident integral membrane proteins was disrupted in HeLa cells with high (level III) C312 expression (**Fig. 2**). TGN46, which localizes to a tight

pericentriolar structure in untransfected cells or cells expressing low levels (level I) of C312 (*stars*), was present in diffuse, vacuolated structures in cells with level III C312 expression (**Fig. 2a-b'**); in some cells expressing extremely high levels, pericentriolar staining was completely absent (not shown). Similarly, HA-tagged furin, which localizes to the TGN in transfected HeLa cells (Bosshart et al., 1994), was mislocalized to a diffuse and "expanded" pericentriolar structure in cells with level III expression of T7-epitope tagged C312 (**Fig. 2e, e'**). These effects were cell-type independent and a function of TGN localization rather than to unrelated functions of the TGN46 and furin luminal domains. This was evident in transiently transfected CHO cell variants by the C312-induced redistribution of the chimeric proteins TGG and TTF, which bear the cytoplasmic domains of TGN38 or furin, respectively, and the luminal and transmembrane domains of an irrelevant protein, Tac (**Fig. 2g-h', j-k'**). Furthermore, AP-1, which associates with the TGN as a peripheral membrane protein, also failed to accumulate in the pericentriolar region in C312-overexpressing cells (**Fig. 2m-n'**). Since TGN46/TGG and furin/TTF localize to the TGN via distinct cytoplasmic targeting signals (Humphrey et al., 1993; Voorhees et al., 1995) and recycling pathways (Ghosh et al., 1998; Mallet and Maxfield, 1999), and AP-1 localization is mediated by independent mechanisms (Page and Robinson, 1995; Seaman et al., 1996), these data show that high level expression of C312 disrupted TGN localization mediated by multiple pathways. Similar results were obtained in COS, MOP8, and NRK cells (unpublished data). Note that the distribution of syntaxin 6 and the cation-independent mannose 6-phosphate receptor did not overlap significantly with the TGN in our untransfected HeLa cells, and thus no change in their distribution could be observed in cells overexpressing C312 (unpublished data).

Three approaches were used to show that the displacement of TGN proteins was due to overexpression of an intact GRIP domain. First, cells were transfected with HA-tagged C312 in which a tyrosine conserved in all GRIP domains was replaced by alanine [C312(Y2187A)]; this fragment failed to localize to the Golgi in transfected cells at any expression level (Cowan et al.,

2002; see also Barr, 1999; Kjer-Nielsen et al., 1999a; Munro and Nichols, 1999). Expression of C312(Y2187A) at levels comparable to those sufficient for the level III phenotype of intact C312 (assessed by semiquantitative analysis of anti-HA fluorescence intensity) failed to disrupt the localization of any analyzed TGN resident protein (**Fig. 2c, f, i, l, o**), indicating that the GRIP domain must be intact to effect TGN disruption. Second, TGN46 mislocalization was observed upon overexpression of a C-terminal fragment of golgin-97, g97.C179, containing an independent GRIP domain (**Fig. 3m, n**). Finally, using semiquantitative IFM analyses in triple stained HeLa cells, we found that comparable levels of C312 expression induced both the mislocalization of TGN46 and the displacement of the endogenous GRIP proteins, tGolgin-1 and golgin-97, from the Golgi (**Fig. 3a-l**). Taken together, these data suggest that saturation of GRIP domain binding sites on TGN membranes or on a GRIP effector molecule results in disruption of the steady state distribution of TGN resident proteins.

To determine whether GRIP domain overexpression interfered with a dynamic process, we tested whether the change in TGN46 distribution was reversible. Because C312 has a short half-life (**Fig. 4a**; see also **Fig. 7**), it could be rapidly depleted by CHX treatment of transfected HeLa cells; treatment for 1-4 hrs increased the fraction of transgene-positive cells with level I staining at the expense of cells with level III staining (**Fig. 4b**), indicating a loss of cells with high level C312 expression. Analysis of this same population of cells for TGN46 localization indicated that the fraction of cells with a wild-type pericentriolar TGN46 staining pattern increased over time of CHX treatment (**Fig. 4c**). Thus, the effect of GRIP domain overexpression on TGN46 distribution is reversible.

Specificity of protein localization defects in GRIP domain overexpressing cells. To determine whether the effects of GRIP domain overexpression were limited to the TGN, we analyzed the distribution of residents of other organelles in cells expressing high levels of C312.

Staining patterns for Golgi stack integral (giantin and mannosidase II; ManII) and peripheral (GM130 and p115) membrane proteins were unaffected by overexpression of C312 (**Fig. 5A, a-h**) or of other GRIP domain-containing fragments (unpublished data). Furthermore, there were no consistent effects on the distribution of actin filaments, cell surface proteins, or markers of the endoplasmic reticulum (ER), ER/Golgi intermediate compartment, early endosomes, or late endosomes/ lysosomes (**Table I**). Pericentriolar staining for β 1,4 galactosyltransferase (GalT), an enzyme that localizes to both the TGN and the trans Golgi cisternae, was disrupted in about half of the cells that overexpressed C312, but not in cells that overexpressed C312(Y2187A) (**Fig. 5B, a-f**). This effect was observed less consistently than for TGN46 or other TGN resident proteins (**Fig. 2**) and did not correlate with C312 expression level (unpublished data), perhaps reflecting the distribution of GalT to both the TGN and Golgi stacks (Nilsson et al., 1993; Rabouille et al., 1995) and cycling between these compartments (Cole et al., 1998; Miesenböck and Rothman, 1995). The results indicate that GRIP domain overexpression affects primarily TGN structure and/or protein localization, with minimal effects on the Golgi stacks and no discernible effects on other membranous organelles.

Rab6 has been implicated as a binding partner for the GRIP domains of tGolgin-1 and golgin-97 (Barr, 1999). Rab6 localization to the Golgi was only marginally affected in C312-overexpressing cells (**Fig. 5g-l** and **Table I**); as for GalT, loss of tight pericentriolar Rab6 staining was observed in only a fraction (25.7%) of C312-overexpressing cells and did not correlate with C312 expression levels. This suggests that Rab6 localization to the Golgi is not dependent on binding of any particular GRIP-domain containing protein, and that excess GRIP domain cannot sequester Rab6 from membranes. Similar results were obtained with a more established GRIP domain binding partner, Arl1 (data not shown), which in yeast is known to regulate – but not to be regulated by – GRIP domain Golgi localization (Panic et al., 2003; Setty et al., 2003).

TGN functional defects induced by GRIP domain overexpression. We next tested whether the alterations in TGN protein distribution observed upon overexpression of GRIP domains underlie TGN functional defects. First, we assayed protein transport from the ER to the plasma membrane using an EGFP-tagged form of the temperature-sensitive ts045 variant of VSV-G (Bergmann et al., 1981; Presley et al., 1997). VSV-G-EGFP misfolds at the restrictive temperature of 39°C and is thus retained within the ER of transfected HeLa cells, producing a reticular pattern throughout the cytoplasm by fluorescence microscopy (**Fig. 6a** and) (Presley et al., 1997). Upon shift to 32°C, accumulated VSV-G-EGFP synchronously exits the ER and traverses the Golgi complex en route to the plasma membrane. VSV-G-EGFP largely accumulates at the Golgi by 30 min and at the plasma membrane by 60 min (**Fig. 6b-c**). Coexpression of VSV-G-EGFP with very high levels of C312(Y2187A), which lacks a functional GRIP domain, had no effect on the kinetics of VSV-G-EGFP transport (compare **Fig. 6a-c** to **d-f**). In cells expressing comparable levels of C312, VSV-G-EGFP staining was also similar to the controls at 39°C and after 30 min at 32°C (**Fig. 6g, h**), indicating normal folding kinetics and ER to Golgi transport. However, after 60-120 min, most C312 expressing cells retained VSV-G-EGFP within the Golgi region (**Fig. 6i, j**), indicating a block in Golgi to plasma membrane transport. By 3hrs, some VSV-G-EGFP reached the plasma membrane in most cells (**Fig. 6k**), indicating that the block was not absolute, but still a substantial proportion of cells displayed prominent pericentriolar fluorescence not observed in the controls. The Golgi to plasma membrane block was dependent on C312 expression level; in cells expressing lower levels of C312, transport proceeded normally such that plasma membrane staining was apparent by 60 min (**Fig. 6l**), whereas in cells expressing extremely high levels of C312, no plasma membrane fluorescence was observed even after 3 hrs (**Fig. 6m**).

As a second test of TGN function, we assessed the ability of proprotein convertases of the furin family to cleave a substrate protein. Furin localizes predominantly to the TGN (Shapiro et al., 1997) where it cleaves target proproteins at dibasic recognition sites (Nakayama, 1997). Pmel17, a

glycoprotein found in melanosome precursors in pigment cells, is cleaved by furin or a related proprotein convertase en route to late endosomes in transfected HeLa cells (Berson et al., 2003). To determine whether furin cleavage is compromised in cells overexpressing GRIP domains, Pmel17 processing was assessed by metabolic pulse/chase and immunoprecipitation in transfected HeLa cells expressing Pmel17 without or with excess C312 or C312(Y2187A). In cells expressing Pmel17 alone, a single ~100kDa band (P1) present in cell lysates from pulse labeled cells matured first to a slower migrating species (P2) by oligosaccharide processing in the Golgi, and then to two faster migrating species (M α and M β) by proprotein convertase cleavage (**Fig. 7b, lanes 1-4**) as observed previously (Berson et al., 2001; Berson et al., 2003) and outlined schematically in **Fig. 7a**. By 4 hrs, most of the P1 and P2 was converted to M α and M β (**lane 4**). The kinetics of processing was virtually unchanged in cells co-expressing C312(Y2187A) (**lanes 9-12**). However, in cells co-expressing C312 at comparable levels (**Fig. 7c**), P2 accumulated and M α and M β were not substantially generated (**lanes 5-8**). The formation of P2, which is resistant to digestion by endoglycosidase H (Berson et al., 2001; Berson et al., 2003), indicates that these cells had no or minimal defects in oligosaccharide processing within the Golgi stack. However, P2 accumulation coupled with M α and M β depletion indicate that proprotein convertase cleavage of Pmel17 was blocked. These data indicate that overexpression of GRIP domains compromises either enzymatic functions or the interactions between enzyme and substrate within the TGN.

Despite these defects, some sorting functions normally ascribed to the TGN appeared to remain intact in C312 overexpressing cells. Transport to late endosomes was unaltered relative to controls as assessed by the steady state localization of several endogenous late endosomal and lysosomal proteins and the transport kinetics of cotransfected chimeric proteins (**Table I**). These results suggest that C312 overexpression affects only certain TGN subdomains or the cycling of only certain cargo.

Ultrastructural defects and mislocalization of TGN46 in cells overexpressing GRIP

domains. To determine the effects of C312 overexpression on TGN morphology in more detail, cells were analyzed by EM. Cells expressing C312 or C312(Y2187A) were enriched by cell sorting following cotransfection with a plasma membrane marker, Tac; by IFM, >90% of sorted cells expressed C312 or C312(Y2187A), the majority at high levels. Sorted cells were incubated with horseradish peroxidase (HRP) for 15-30 min at 37°C as a marker of fluid phase endocytosis prior to fixation, and then analyzed by EM either for morphology after embedding in epon; or by immunoEM after indirect immunogold labeling of ultrathin cryosections with antibodies to HA, HRP, and/or TGN46.

HeLa cells transfected with C312(Y2187A) displayed a relatively normal morphology, similar to untransfected cells, with intact, flattened pericentriolar Golgi cisternae (**Fig. 8a**). Cells transfected with C312 were more heterogeneous, but displayed general defects in Golgi morphology. Intact Golgi cisternae were difficult to find in most C312-expressing cells. They were replaced by large, pericentriolar vacuolated structures (**Fig. 8b, c**). The vacuoles varied in number, reflecting an apparent proliferation of Golgi/TGN rather than simply engorged cisternae. The structures often displaced a large fraction of the cytoplasm, and some of the vacuoles contained internal membrane sheets. Multivesicular endosomes (mve) were also more abundant in cells expressing C312 than those expressing C312(Y2187A) (e.g., see **Fig. 8c**). These data indicate that GRIP domain overexpression results in a large scale disruption of Golgi/TGN architecture and alterations in the abundance of late endosomes.

Immuno-EM analyses (**Fig. 9**) revealed the basis for the phenotypes observed by IFM. In cells expressing high levels of C312(Y2187A), TGN46 was localized as expected to cisternae and tubulovesicular structures at the trans side of the Golgi stack (**Fig. 9A**). A similar pattern was observed in cells expressing low levels of C312, in which TGN46 and C312 were colocalized in

these structures (unpublished data). In cells expressing high levels of C312, however, TGN46 was instead detected primarily in two types of membrane compartments. One type comprised large vacuolated structures with few internal membranes (**Fig. 9B, C**), likely corresponding to those observed by conventional EM (**Fig. 8**). These structures were labeled abundantly on the cytoplasmic face by anti-HA antibodies detecting the C312 transgene (anti-HA labeling was also observed throughout the cytoplasm and in large, electron dense inclusions; unpublished data). The second class of compartments comprised multivesicular structures with numerous intraluminal vesicles (**Fig. 9D-F**). These structures were mve, since they were also labeled by HRP following internalization for 15 or 30 min (**Fig. 9E, F**). TGN46 labeling in mve was found both on the limiting and intraluminal membranes, suggesting targeting for degradation in lysosomes. Since TGN46 normally bypasses the mve and trafficks through recycling endosomes en route to the TGN (Ghosh et al., 1998), these data suggest that GRIP domain overexpression results in TGN46 mistargeting to both late endocytic organelles and enlarged, vacuolated Golgi-derived structures, thus interfering with the normal delivery of TGN46 from the endocytic pathway to the TGN.

DISCUSSION

Mammalian GRIP protein function has remained undefined, and a role for the yeast GRIP protein, Imh1p, in Golgi maintenance is inferred only from indirect genetic interactions. Here we have shown that overexpression of isolated GRIP domains in cultured mammalian cells results in specific disruption of TGN morphology, protein localization, and function. The data provide evidence that at least one GRIP protein and/or GRIP ligand functions in TGN maintenance, likely by regulating recycling from endosomes.

Specificity of GRIP-domain-dependent disruption of vesicular transport. The displacement of endogenous GRIP proteins by expression of increasing doses of exogenous GRIP domains had been shown previously (Kjer-Nielsen et al., 1999a; Kjer-Nielsen et al., 1999b), but this is the first report to describe a concomitant redistribution of all TGN resident proteins. That TGN disruption required a functional GRIP domain was confirmed by (1) induction of a similar phenotype by hyper-expression of all GRIP-domain containing fragments of tGolgin-1 and golgin-97, (2) the inactivity of N-terminal tGolgin-1 fragments that lack the GRIP domain, and (3) the inactivity of a tGolgin-1 GRIP domain fragment with a mutation in the critical tyrosine residue. The innate instability of C-terminal fragments of tGolgin-1 (see **Figs. 4, 7**) and the requirement for high level expression of GRIP domain-containing fragments to disrupt TGN localization may explain why this phenotype was not previously noted. Although TGN homeostasis was affected by overexpression of both tGolgin-1 and golgin-97 GRIP domains, subtle differences in the IFM staining pattern of the residual TGN and in the effects on some Golgi stack residents suggest some specificity in the function of individual GRIP domains.

With few exceptions, the disrupting effects of GRIP domain overexpression were limited to the TGN, with the strongest effects on TGN46 distribution. The effects did not extend to other organelles, consistent with the localization of GRIP proteins to the TGN (Brown et al., 2001;

Gleeson et al., 1996; Luke et al., 2003). Effects on the Golgi stack were mixed. The distribution of most Golgi resident proteins by IFM was unaltered in GRIP overexpressing cells relative to controls, and Golgi function was largely unaffected based on the pericentriolar accumulation of ER-released VSV-G and on the largely unchanged kinetics of acquisition of Golgi modifications to Pmel17. Other effects on the Golgi may have been secondary to TGN disruption, perhaps as a consequence of cycling of stack residents through the TGN (Johnston et al., 1994). Two Golgi stack residents that also localize to the TGN (Martinez et al., 1994; Rabouille et al., 1995), GalT and Rab6, were redistributed in only a fraction of cells that expressed GRIP domain fragments at a threshold level sufficient to disrupt TGN46 distribution and not in a manner that correlated with GRIP expression level. The failure to observe characteristic Golgi stacks by EM in cells overexpressing GRIP domains was likely due to obscuring by the bloated TGN membranes; and/or to slight Golgi cisternal dilation proximal to the centriole. Finally, the redistribution of the early Golgi v-SNARE, GS28, in cells overexpressing the tGolgin-1-derived C312 (unpublished data) may, as for Rab6 and GalT, reflect a more general distribution of this vSNARE throughout the Golgi and TGN (Nagahama et al., 1996).

How might TGN dynamics be disrupted by GRIP domain overexpression? The expansion of membrane observed by EM and the failure to sort VSV-G from the Golgi to the plasma membrane suggest a defect in TGN export, consistent with in vitro budding of tGolgin-1-bound membranes from purified Golgi stacks (Gleeson et al., 1996). However, disrupted Golgi export is likely a secondary consequence of a primary failure to properly localize TGN resident proteins for several reasons. First, defective TGN export would not explain the redistribution of TGN resident proteins to peripheral structures. Second, this redistribution occurred at GRIP expression levels similar to those required to displace tGolgin-1 from the Golgi, suggesting that TGN46 mislocalization was a primary effect of competition for GRIP domain binding sites. Third, TGN46 was largely mislocalized to endosomes, from which TGN46 is normally recycled to the TGN (Ghosh et al., 1998; Mallet and Maxfield, 1999). We thus favor the interpretation that competition for GRIP

domain binding sites interferes with the recycling of TGN46 from endosomes to the TGN (**Fig. 10**). The secretory defect could then result secondarily from the depletion of factors from the TGN that follow a similar recycling pathway and that are required for subsequent budding of plasma membrane-bound cargo, such as cargo recruitment proteins (Rojo et al., 1997) or v-SNAREs (Gurunathan et al., 2000; Salem et al., 1998; Springer and Schekman, 1998) (**Fig. 10**).

Displacement of TGN resident proteins that follow a different recycling pathway, such as GalT and furin, might also be secondary to changes in TGN architecture. This model would explain our failure to observe redistribution of endosomal residents (**Table 1**), which rely on distinct cargo recruitment proteins and SNAREs with distinct recycling pathways. Our model predicts that only the post-endocytic endosome-to-TGN recycling step would be blocked by GRIP domain overexpression, consistent with qualitative assessments that failed to detect defects in internalization, recycling, and late endocytic delivery of several internalized cargo proteins (unpublished data).

Potential mechanisms of TGN disruption by GRIP domain overexpression.

What might be the molecular basis for the effects of GRIP domain overexpression? One potential explanation is competitive displacement from GRIP domain binding sites of endogenous GRIP proteins that are required for TGN maintenance. The GRIP proteins may play a direct role in maintaining TGN homeostasis, or serve as regulators of an effector (or effectors) of TGN maintenance such that overexpression of the GRIP domain would saturate binding sites and block effector function. The structural similarity of GRIP proteins to tethering factors involved in membrane fusion events at other sites within the secretory and endosomal system (reviewed in (Pfeffer, 1996; Pfeffer, 1999) or to components of the “Golgi matrix” required for stacking Golgi cisternae (Warren and Shorter, 2002) would be consistent with such a function. A second potential explanation for the effects is that overexpressed GRIP domains sequester GRIP domain ligands that function in TGN maintenance. Such ligands may interact with additional effectors, and thus their sequestration by excess GRIP domains could block functions in which GRIP proteins are not

directly involved. The best candidate for such a ligand is Arl1. GTP-bound Arl1 associates with GRIP domains and other effectors *in vitro* (Lu et al., 2001; Panic et al., 2003; Setty et al., 2003; Van Valkenburgh et al., 2001) and in yeast is required for GRIP protein recruitment to the TGN (Panic et al., 2003; Setty et al., 2003). Moreover, the vacuolization of the Golgi and TGN observed here is similar to that observed upon overexpression of a predicted GTP-locked form of Arl1 (Lu et al., 2001; Van Valkenburgh et al., 2001), and another putative Arl1 effector in yeast, the VFT complex (Panic et al., 2003), has been implicated in regulating recycling from endosomes to the TGN (Siniosoglou et al., 2001, 2002). Another candidate GRIP ligand, Rab6 (Barr, 1999), is a known effector of endosome to TGN recycling (Mallard et al., 2002) but less likely to be responsible for the observed phenotype; a GTP-locked form of Rab6 induces TGN46 redistribution (Martinez et al., 1994), but only at extremely high levels of expression and the effect is phenotypically distinct from that induced by GRIP overexpression (unpublished data). GRIP domains might also conceivably sequester lipid ligands, such as phosphatidylinositol phosphates which are known to regulate the morphology and function of the TGN and Golgi (Godi et al., 1999; Munro, 1998). Biochemical analyses of GRIP domain binding to Golgi membranes should help to dissect potential GRIP effectors and the mechanism of GRIP overexpression-induced TGN disruption.

ACKNOWLEDGEMENTS

The authors wish to thank Sean Jordan for technical support, B. Wendland, C. Machamer, and C. Burd for critical review of the manuscript, M. I. Greene for support of DG, S. Munro, M. A. Lemmon and C. G. Burd for helpful discussions, H. Pletscher for help with cell sorting, and all of the investigators who graciously sent us reagents, particularly E. Berger, J. S. Bonifacino, T. Braulke, M. Fritzler, M. Fukuda, N. Gonatas, H.-P. Hauri, W. Hong, L. Johannes, J. Lippincott-Schwartz, F. Maxfield, K. Moremen, S. Munro, and V. Ponnambalam. This work was primarily supported by American Cancer Society Research Project Grant RPG-00-238-01-CSM to MSM and by National Science Foundation grant NSF-DBI-0099706 to JMM. AY was supported in part by grant # DAMB17-01-1-0365 from the U.S. Dept. of the Army. SS was partly supported by training grant 5 T32 CA 09140 from the National Cancer Institute. DG was supported by grants from the Lucille P. Markey Charitable Trust and the National Multiple Sclerosis Society.

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FIGURE LEGENDS

Figure 1. Saturation of tGolgin-1 C312 localization in HeLa cells. HeLa cells transiently transfected with plasmid expressing HA-tagged C312 were analyzed 2 days later by IFM with anti-HA and RRX-conjugated secondary antibodies. *a-c*, examples of cells with different expression levels of C312 classified as levels I, II, and III as indicated. *d*, comparison of semiquantitative total cell fluorescence levels with phenotypic classification. Total fluorescence from individual cells (in arbitrary units) was measured using OpenLab software as indicated in Materials and Methods, and plotted on the y-axis. Each dot represents the measurement from a single cell; the bar represents the median expression level from all cells.

Figure 2. Overexpression of C312 disrupts the localization of TGN resident proteins. HeLa cells (*a-f, m-o*) or stable transfectants of TRvb-1 cells expressing TGG (*g-i*) or TTF (*j-l*) were transiently transfected with C312 or C312(Y2187A), as indicated, and analyzed by IFM using antibodies to the indicated proteins and to the HA tag (' columns) and appropriate secondary RRX- and FITC-conjugated secondary antibodies. In *d-f*, cells were cotransfected with furin-HA (50-100ng/ 6 well dish) and T7-epitope-tagged C312 (5-7 μ g/ 6 well dish); anti-HA was used to detect furin and anti-T7 to detect C312. Panels *a, d, g, j*, and *m* focus on cells with low C312 expression (level I), and panels *b, e, h, k*, and *n* focus on cells with high expression (level III); shown in panels *c, f, i, l*, and *o*, are selected cells in which semiquantitative analyses showed levels of HA staining comparable to those in panels *b, e, h, k*, and *n*.

Figure 3. Parallel displacement of TGN46 and endogenous GRIP domain proteins at similar expression levels of C312. *a-j*. HeLa cells that were transiently transfected with C312 were analyzed by three-color IFM using rat anti-HA, mouse anti-tGolgin-1, and sheep anti-TGN46

with AMCA-, RRX- and FITC-conjugated species-specific antibodies, respectively. *a-i*, representative images of staining patterns for HA-C312 (*a, d, g*), TGN46 (*b, e, h*) and tGolgin-1 (*c, f, i*) obtained with C312 expressed at level I (*a-c*), II (*d-f*) and III (*g-i*). *j*, semiquantitative analyses of total cell expression level of AMCA fluorescence (representing C312) in cells characterized as having a characteristic tight pericentriolar Golgi staining pattern (**intact**), diffuse paranuclear staining (**diffuse**), or diffuse cytoplasmic distribution (**cytoplasmic**) for TGN46 and endogenous tGolgin-1. C312 expression is plotted in arbitrary units on a log scale on the y-axis; circles represent values for individual cells, and bars represent the median of all analyzed cells. *k, l*, cells transiently transfected with C312 were analyzed by IFM using antibodies to HA (*k*) and golgin-97 (*l*). *m, n*, cells transiently transfected with g97-C179 were analyzed by IFM using antibodies to HA (*m*) and to TGN46 (*n*).

Figure 4. Reversibility of TGN46 displacement induced by GRIP domain overexpression.

a. HeLa cells transiently transfected with C312 or C312(Y2187A) or control untransfected cells were metabolically labeled with ³⁵S-methionine/cysteine for 30 min and then chased for 0, 1, 2, or 4 hrs. C312 or C312(Y2187A) was immunoprecipitated from cell lysates at each time point using anti-HA antibodies, fractionated by SDS-PAGE, and total C312 levels were determined by phosphorimaging analysis of the 40 kDa band that was absent in the controls (see Fig. 7). The amount at time 0 was set to 100%, and the per cent remaining at each time point is plotted. Shown is a representative of 3 experiments. *b, c*. HeLa cells from the same well transiently transfected with C312 were treated with 10 µg/ml CHX for 0, 2 or 4 hrs as indicated, fixed, and then analyzed by IFM using antibodies to the HA-epitope (*b*) and to TGN46 (*c*). *b*. The percentage of cells in a representative experiment that were positively stained with anti-HA (n=238 to 269 per time point in this experiment) were characterized for phenotypic expression level of C312 expression. *c*. The percentage of cells in the same experiment that were positively stained with anti-HA were

characterized for phenotypic appearance of TGN46 staining; “intact” TGN46 indicates a tight Golgi ribbon as in **Fig. 2a**.

Figure 5. Localization of Golgi stack residents in cells overexpressing C312. HeLa cells transiently transfected with C312 were analyzed by IFM using antibodies to the HA-epitope (to detect C312) and to endogenous Golgi markers as indicated. *Aa-h*, cells overexpressing C312 and co-stained for giantin, mannosidase II (ManII), GM130, or p115. Examples are representative of all cells examined. *B*, cells co-stained for galactosyltransferase (GalT; *a-f*) or Rab6 (*g-l*). In approximately 46% of analyzed C312-overexpressing cells ($n=369$ over 3 experiments), the GalT staining pattern was similar to untransfected cells in the same field (*a, b*) and to cells transfected with C312(Y2187A) (*e, f*), whereas in the other 54%, GalT staining was diffuse (*c, d*). A similar disparity was seen for Rab6; in approximately 74% of analyzed C312-overexpressing cells ($n=175$ over 2 representative experiments), Rab6 staining was similar to controls (*g, h* vs. *k, l*) and in 26%, Rab6 staining was diffuse (*i, j*).

Figure 6. GRIP domain overexpression affects Golgi to plasma membrane transport. HeLa cells were transiently transfected with expression vectors for VSV-G-EGFP (2.5 μ g/ 6 well dish) and either vector alone (*a-c'*), C312(Y2187A) (*d-f'*) or C312 (*g-m'*) at 5 μ g/ 6 well dish. Cells were grown at 39°C for 1 day and then fixed either before (*a, d, g*) or after shifting to 32°C for the indicated times. Cells were analyzed by fluorescence microscopy after immunostaining with anti-HA and RRX-conjugated secondary antibodies (‘ panels); EGFP was visualized directly (non-‘ panels). Panels *g-k'* show examples of cells with similar anti-HA staining intensities as those shown in panels *d-f'*; *l* and *l'* show an example of a cell with low C312 expression, and *m* and *m'* show an example of a cell with very high C312 expression. All ‘ panels were taken at the same exposure time on samples prepared and analyzed on the same day. The “spotty” appearance of VSV-G-EGFP localization in *a, d* and *g* may result from a fixation artifact.

Figure 7. GRIP domain overexpression affects proprotein convertase activity on a

substrate protein, Pmel17. *a.* Schematic diagram of Pmel17 primary structure and processed forms, essentially as described and shown in ref. (Berson et al., 2001). *b.* HeLa cells were transiently transfected with expression vectors for Pmel17 (10 µg/ 10cm dish) and either vector alone (lanes 1-4), C312 (lanes 5-8) or C312(Y2187A) (lanes 9-12) at 39 µg/ 10cm dish. Two days post-transfection cells were harvested and pulse labeled with ³⁵S-methionine/cysteine for 30 min, and then chased for the indicated times. Pmel17 was immunoprecipitated from cell lysates at each time point, fractionated by SDS-PAGE, and visualized by phosphorimaging analysis. *c.* Anti-HA immunoprecipitates from the same samples analyzed in the same way. Only the relevant portion of the gel encompassing C312 or C312(Y2187A) transgene products is shown.

Figure 8. GRIP domain overexpression disrupts Golgi morphology. HeLa cells transfected with expression vectors for Tac and for A. C312(Y2187A) or B, C, C312 were sorted for Tac cell surface staining, exposed to HRP for 15-30 min at 37°C, and then fixed and embedded in epon for conventional EM analyses. Note the flattened stacks of Golgi complex (Gc) cisternae in A and their absence in B and C. m, mitochondria; n, nucleus. In C, stars (*) are placed next to multivesicular bodies, which were abnormally abundant in most profiles from C312-transfected cells. Bars, xxx microns.

Figure 9. TGN46 localizes to vacuoles and to multivesicular bodies in C312-

overexpressing cells. HeLa cells transfected with expression vectors for Tac and for A. C312(Y2187A) or B-F C312 were sorted for Tac cell surface staining. Positive cells were exposed to HRP for 15-30 min at 37°C, then fixed, and ultrathin cryosections were labeled with antibodies to HA, TGN46, and or HRP and gold-conjugated secondary antibodies for immuno-EM analyses. A-

C, sections were labeled with anti-HA and 10-nm gold-conjugated anti-mouse immunoglobulin, and anti-TGN46 and 5-nm gold-conjugated anti-sheep immunoglobulin. Arrowheads in *B*. and *C*. point to 5-nm gold particles. Note the labeling of the trans face of the Golgi complex by both anti-HA and anti-TGN46 in *A*, and the dense labeling of vacuoles with anti-HA in *B* and *C*. These examples show TGN46 in the vacuolated structures. *D-F*, sections were labeled with anti-HRP and 10-nm gold-conjugated anti-rabbit immunoglobulin, and anti-TGN46 and 5-nm gold-conjugated anti-sheep immunoglobulin. Arrowheads point to 5-nm gold particles (TGN46) in multivesicular endosomes (mve) that are also labeled by anti-HRP (10-nm gold).

Figure 10. Model for functional effects of GRIP domain overexpression. *a*, simplified model of vesicular traffic in and out of the TGN in HeLa cells, showing relevant endosomal compartments, the TGN, and the Golgi stack; early and recycling endosomes are grouped together for simplicity. Cargo following the indicated pathways between organelles are boxed. GRIP proteins or GRIP interacting molecules are shown to facilitate recycling of proteins from early/recycling endosomes to the TGN. *b*, perturbation of these pathways by overexpression of GRIP domain proteins.

Table I. Extent of protein mislocalization caused by the overexpression of the mouse tGolgin-1 C312 fragment

	C312*	C312(Y2187A)*
<i>ER</i>		
Calnexin	No	No
<i>Intermediate compartment</i>		
ERGIC-53	No	No
<i>Golgi stack</i>		
Giantin	No	No
GM130	No	No
p115	No	No
Mannosidase II	No	No
<u>Galactosyl Transferase</u>	46% affected	No
<u>Rab6</u>	26% affected	No
<i>Trans-Golgi network</i>		
<u>TGN46</u>	Yes	No
<u>Furin-HA (transgene)</u>	Yes	No
<u>TGG (transgene)</u>	Yes	No
<u>TTF (transgene)</u>	Yes	No
<u>AP-1</u>	Yes	No
<u>tGolgin-1</u>	Yes	No
<u>Golgin-97</u>	Yes	No
<i>Early endosome</i>		
EEA1	No	No
Transferrin receptor	No	No
Internalized transferrin	No	No
<i>Late endosome/ lysosome</i>		
Lamp1	No	No
Lamp2	No	No
CD63	No	No
TTL1 (transgene)	No	No
Tac-DKQTLL (transgene)	No	No
<i>Cell surface</i>		
MHC class I	No	No
Tac (transgene)	No	No

* Table tabulates whether changes were observed in the IFM pattern for the indicated marker proteins upon level III expression of either C312 or C312(Y2187A), as indicated. "No" indicates no alteration in >95% of examined cells with level III expression pattern in at least three separate experiments; "Yes" indicates alteration in >95% of examined cells with level III expression pattern. For Rab6 and GalT, patterns were changed in a fraction of cells (alterations did not correlate with expression level of C312). TGG, TTF, and TTL1 are chimeric proteins containing the luminal domain of Tac and the cytoplasmic domains of TGN38, furin, and lamp1 respectively; TGG contains the TGN38 transmembrane domain, whereas TTF and TTL1 contain the Tac transmembrane domain. Tac-DKQTLL contains the entire Tac coding sequence appended with the di-leucine-based sorting signal of CD3 γ .

Figure 1

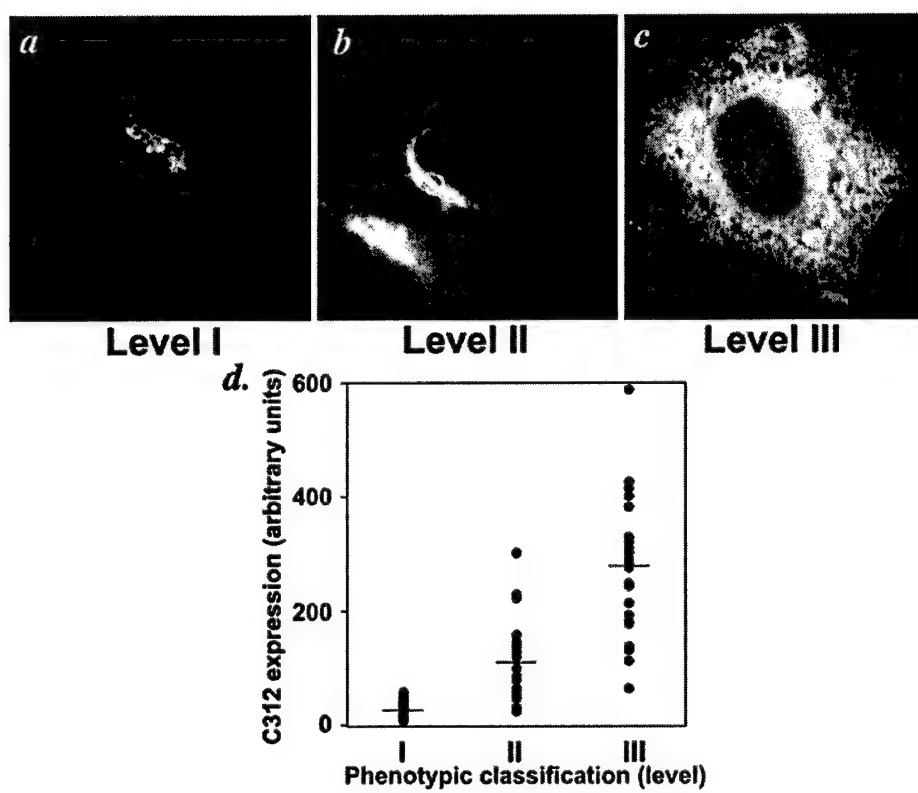


Figure 2

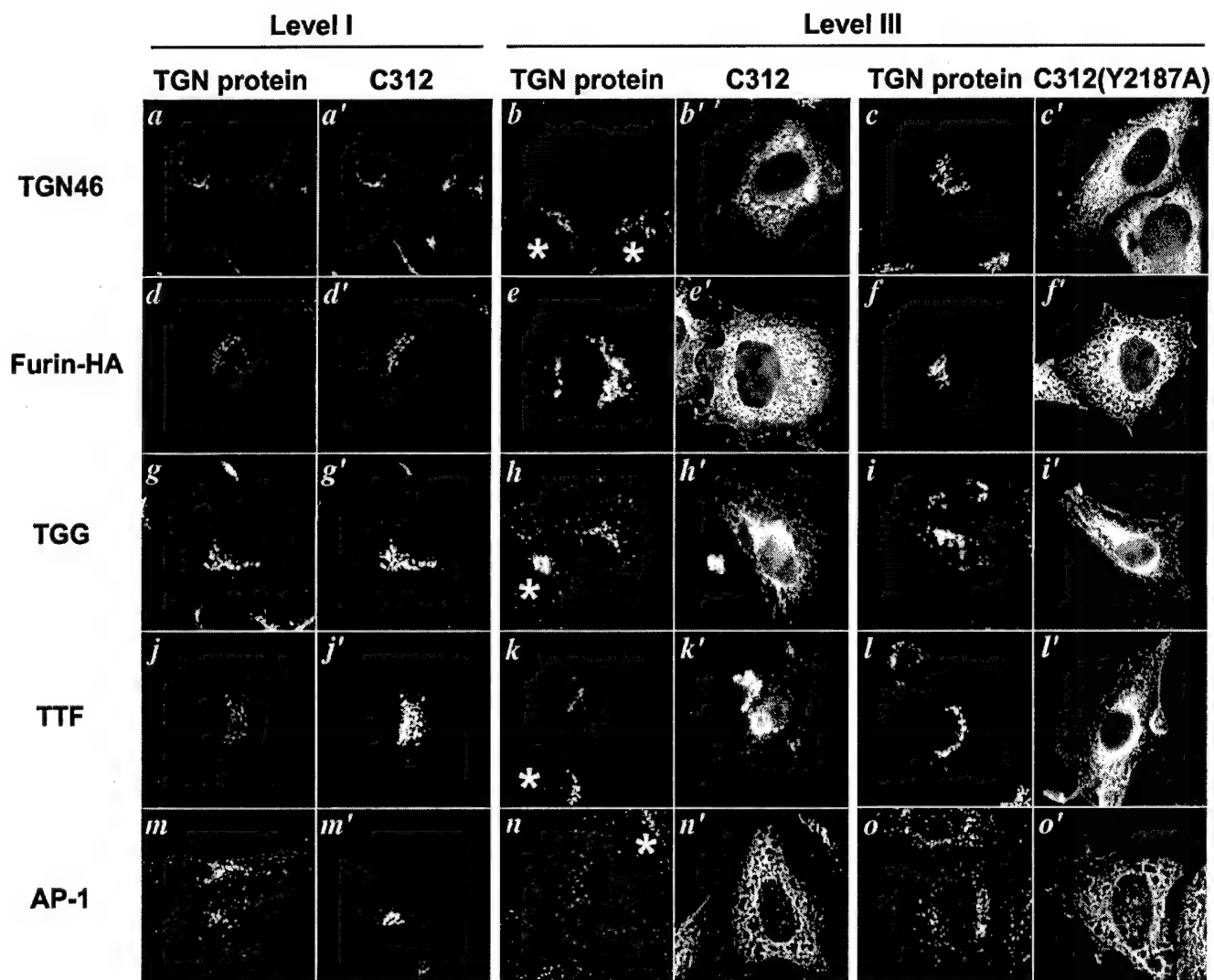


Figure 3

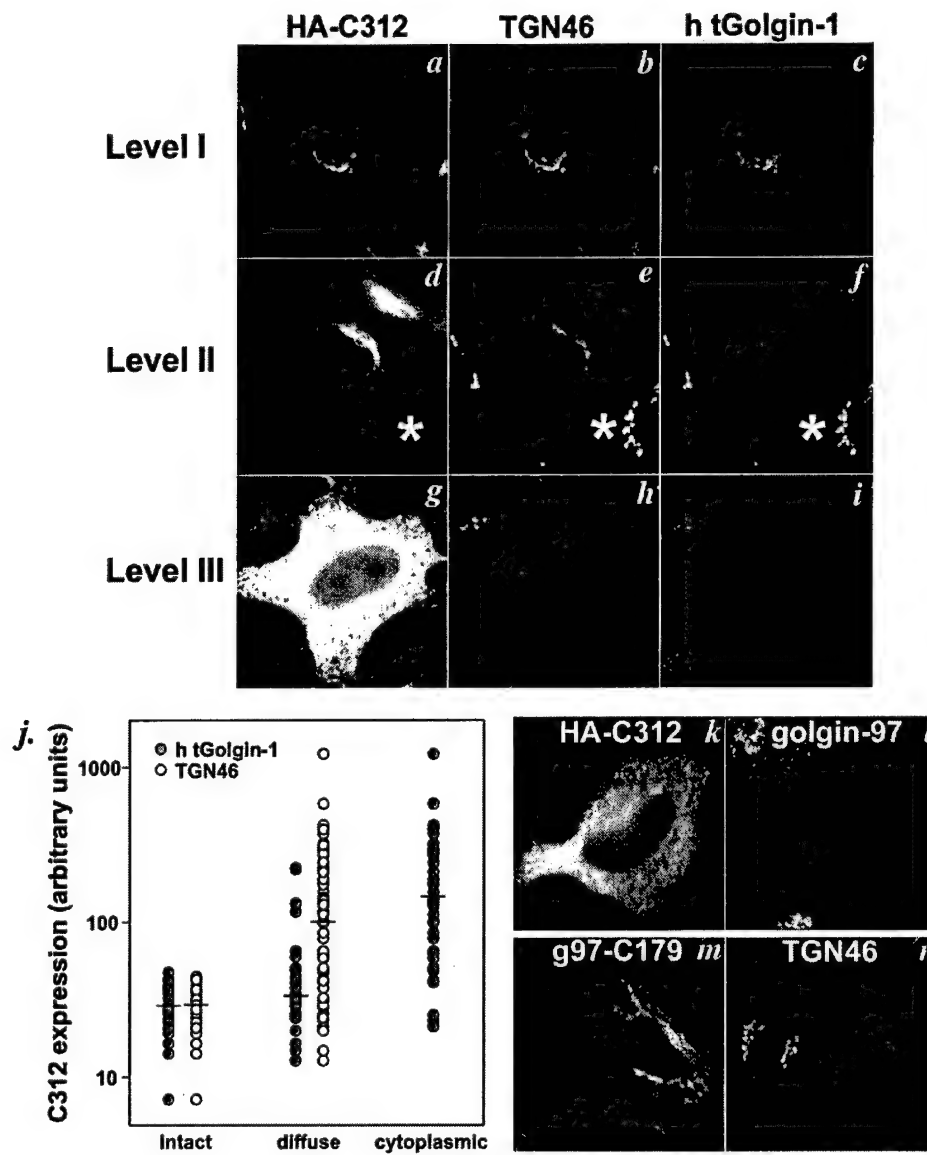


Figure 4

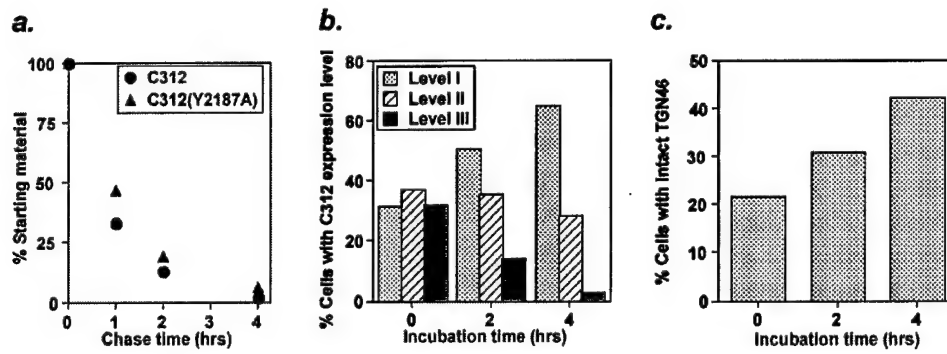


Figure 5

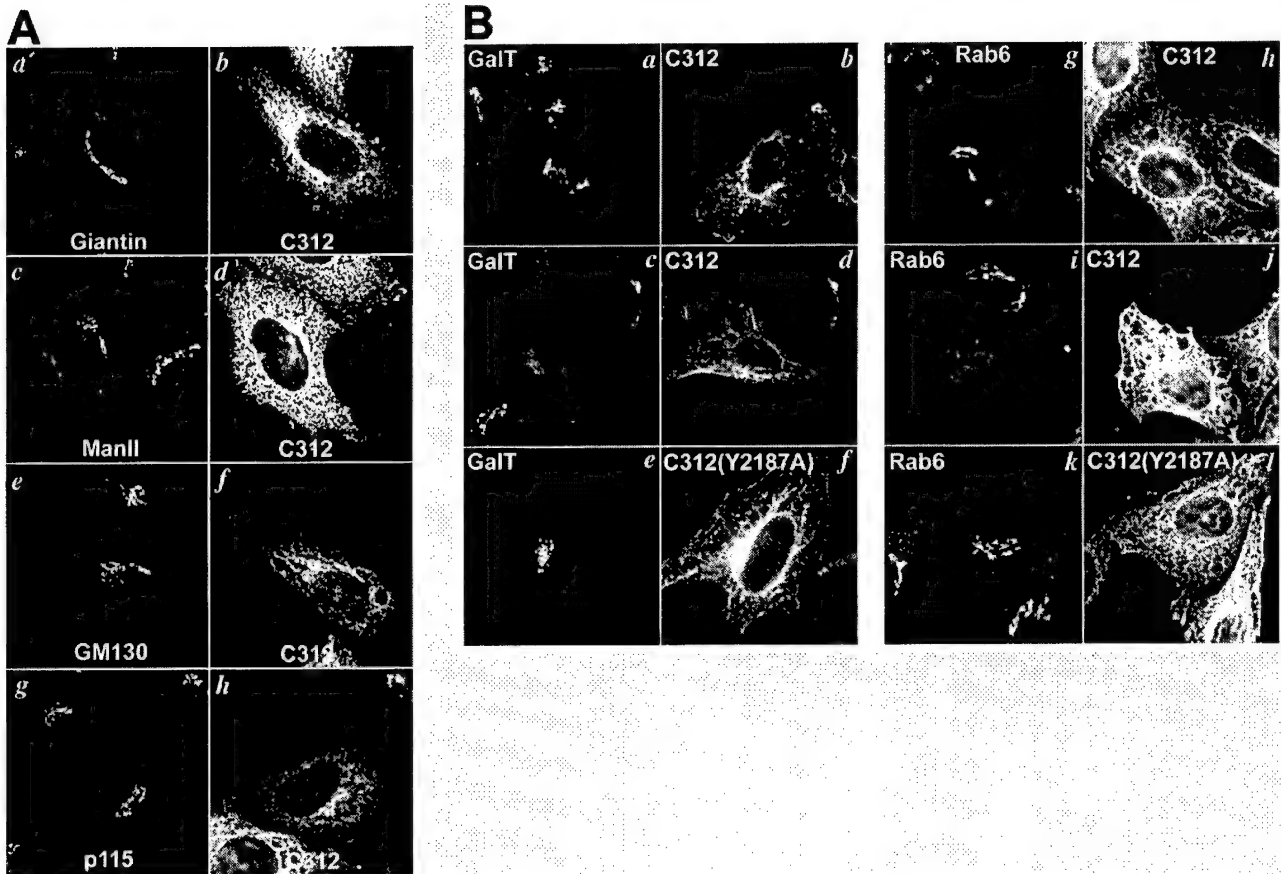


Figure 6

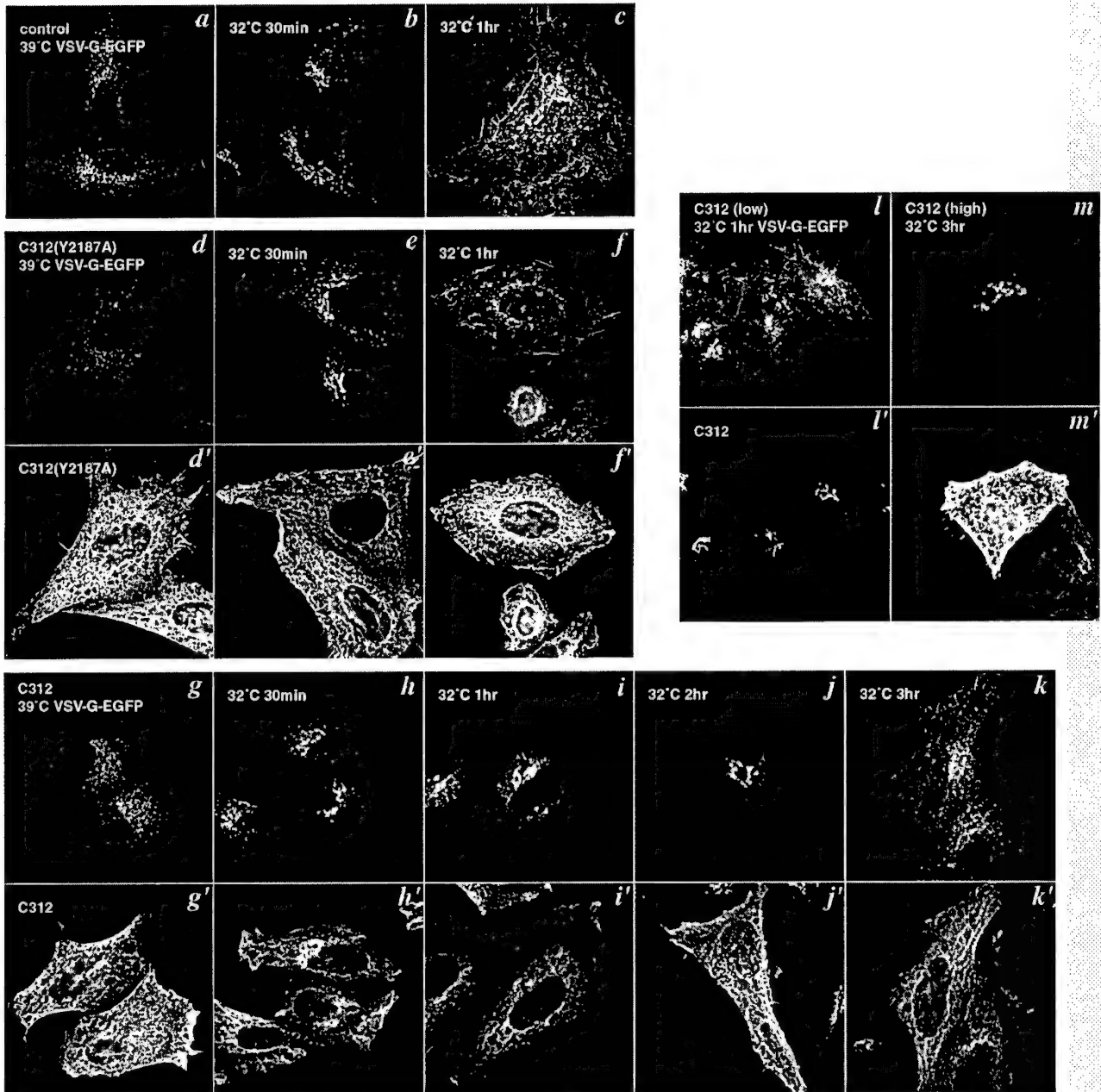


Figure 7

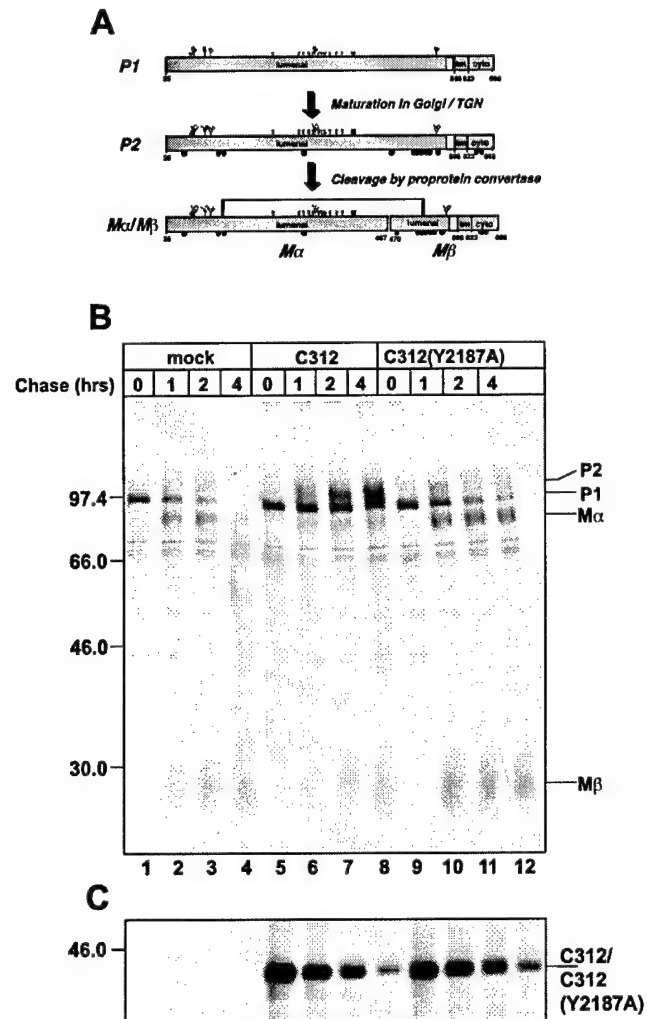


Figure 8

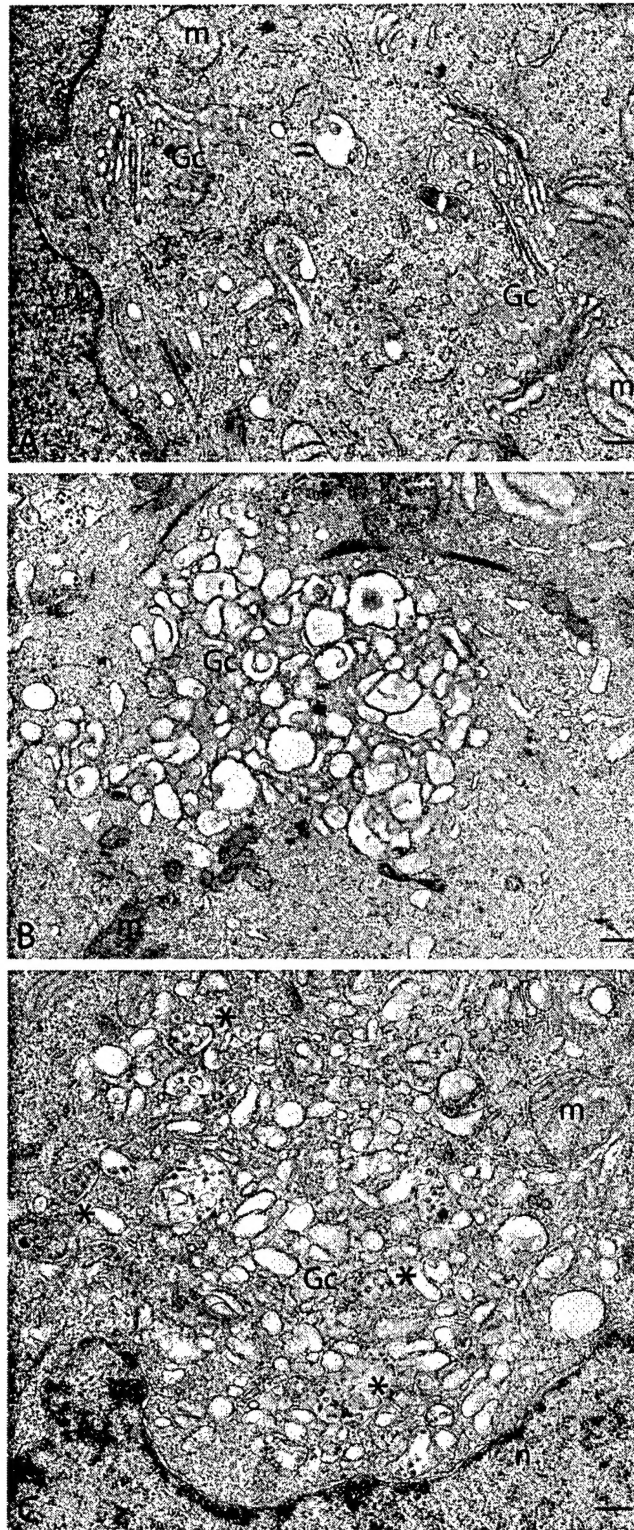


Figure 9

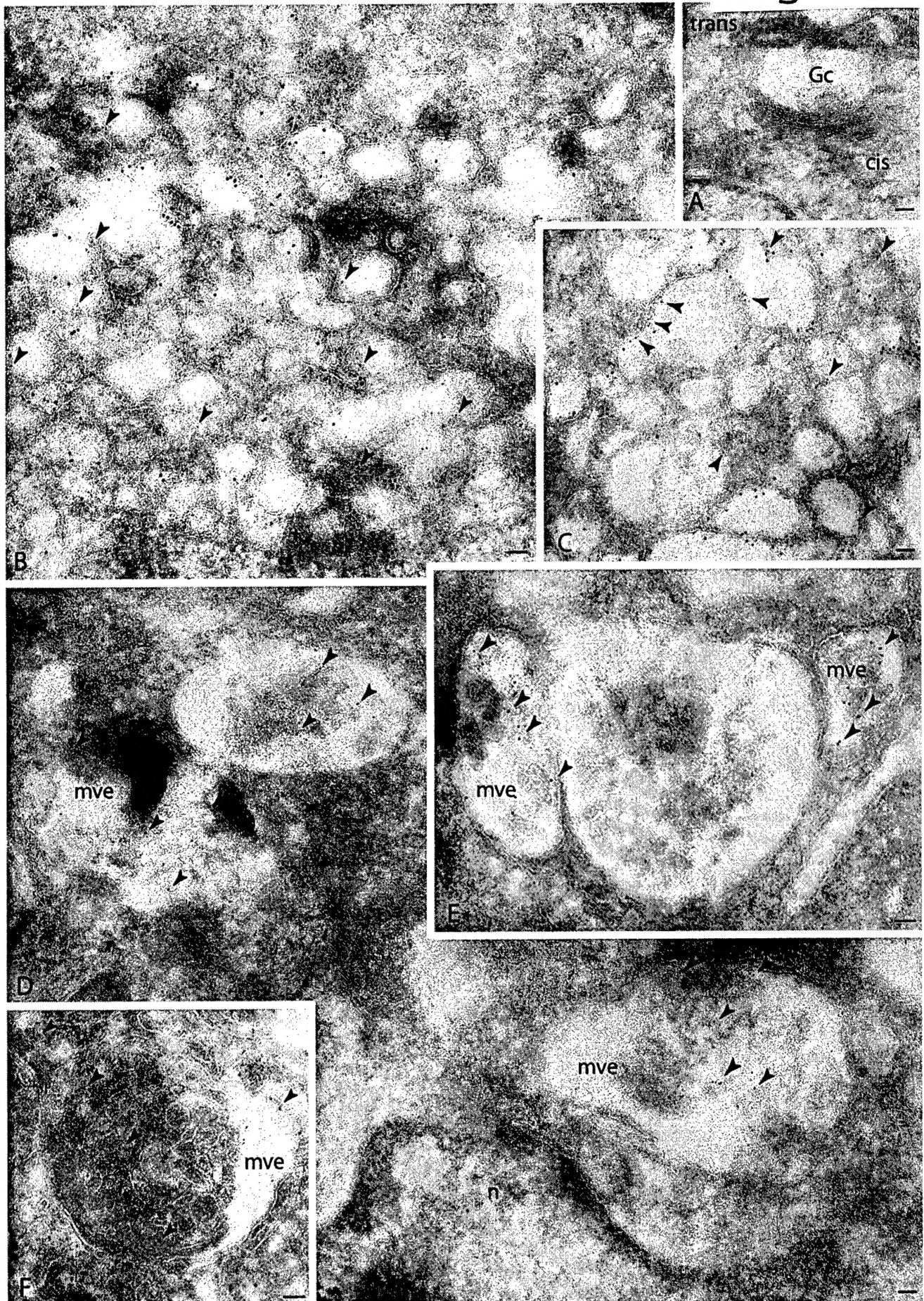
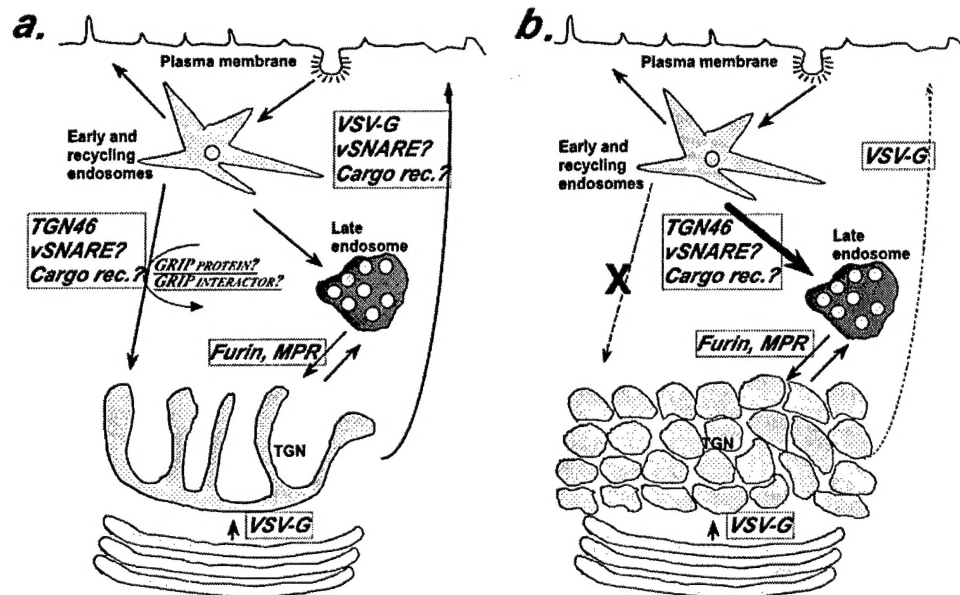


Figure 10



The 76th Annual Meeting of the Japanese Biochemical Society ('03)
October 15-18, 2003. Yokohama, Japan.

Characterization of the function of TGN-localized peripheral membrane protein, tGolgin-1.

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Abstract:

tGolgin-1 (also known as trans golgi p230) is a large Golgi-associated peripheral membrane protein of unknown function. Here, we report the effect of specific gene silencing of tGolgin-1 using RNA interference (RNAi) in cultured cells. Upon transfection of HeLa cells with siRNA duplexes, the expression of tGolgin-1 was drastically reduced as assessed by immunofluorescence microscopy (IFM) and immunoblotting. Interestingly, the distribution of the Golgi and TGN was disrupted by gene silencing of tGolgin-1, resulting in the loss of a pericentriolar ribbon and the appearance of punctate, dispersed structures, which were localized in close proximity to ER exit sites and retained the characteristic *cis/trans* polarity of the Golgi complex. This phenotype is similar to that observed in cells either in which microtubules are disrupted by nocodazole-treatment or in which dynein/ dynactin function is disturbed by dynamitin-overexpression. Importantly, microtubules remain intact in RNAi cells, and the peripheral Golgi-like structures align along the microtubules. These data suggest that tGolgin-1 is required for minus-end-directed motility of Golgi elements along microtubules and thus to maintain Golgi/TGN structure in close proximity to the MTOC. Now we further examine the vesicular motility in living cells.

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